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AN INVESTIGATION OF THE IMPORTANCE OF SOMATIC MUTATIONS AND BASIC RESIDUES IN THE BINDING OF ANTIPHOSPHOLIPID ANTIBODIES TO CARDIOLIPIN

by

Ian Philip Giles

Department of Medicine

University College London

A Thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Science

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ABSTRACT

Sequence analysis of human antiphospholipid antibodies (aPL) has shown that their binding properties are derived from the accumulation of replacement mutations and basic residues in their complementarity determining regions (CDRs). This thesis describes the effects of particular CDR motifs, often sites of somatic mutation and/or containing arginine residues, on the binding to cardiolipin (CL) of two human IgG monoclonal aPL, IS4 and CL24.

Following sequence analysis of IS4 and CL24 a transient expression system was used to express whole IgG containing variants of IS4 and CL24 produced by chain shuffling experiments with three other monoclonal antibodies, UK4, B3 and 33H11. The light chain sequences (V_L) of four of these five mAbs were encoded by the same germline V_L gene and hence differed only in their pattern of somatic mutations. The heavy chain (V_H) of IS4 was dominant in conferring the ability to bind CL in a direct ELISA, whilst the identity of the paired V_L was important in determining the strength of CL binding. Computer-generated models revealed surface exposed arginine residues in the CDRs of IS4V_H and those V_L sequences that were particularly favourable for binding to CL.

Seven new V_L sequences and six new V_H sequences were produced by using CDR exchange and site-directed mutagenesis to alter the patterns of somatic mutation and arginine residues in the wild-type V_L and V_H sequences of these antibodies. Alteration of specific arginine residues in B3V_L CDR1 and IS4V_H CDR3 led to a significant reduction in CL binding. It was concluded that it is not just the presence but precise locations of specific arginine residues in the CDRs of pathogenic aPL, which are important in determining binding affinity for CL.

In order to produce larger quantities of these expressed V_H/V_L combinations, a stable expression system was developed. Three stable cell lines were produced, expressing IS4V_H with three different V_L sequences. The IgG produced showed the same CL binding characteristics as those produced in the transient system.

DEDICATION

I dedicate this thesis to: - Claire - whose patience, support and love made this work possible; Ella and Tom - who keep my feet on the ground and make it fun to come home each day; and to the memory of my mother may she rest in peace.

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Finally, I would like to thank my parents for all the love and support that they have given me over the years.

DECLARATION

The work described in this thesis was carried out by the author unless otherwise stated.

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ABBREVIATIONS

AA	Amino acid
Ab	Antibody
aCL	Anticardiolipin antibodies
Ag	Antigen
ANA	Anti-nuclear antibodies
APC	Activated protein C
APCs	Antigen presenting cells
aPL	Antiphospholipid antibodies
APS	Antiphospholipid Syndrome
ARD	Autoimmune rheumatic disease
AS	Atherosclerosis
β_2GPI	β_2-Glycoprotein I
BFP-STS	Biologically false positive serological tests for syphilis
BIC	Bicarbonate buffer
bp	Base pairs (of DNA)
BSA	Bovine serum albumin
C	Constant region
CAPS	Catastrophic APS
CDR	Complementarity-determining region
C_H	Constant region of heavy chain
CL	Cardiolipin
C_L	Constant region of light chain
CTL	Cytotoxic lymphocyte
D	Domain
dhfr	Dihydrofolate reductase gene
DM	Domain deleted mutant
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds	Double stranded
DVT	Deep vein thrombosis
EC	Endothelial cell

EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen binding fragment of antibody
FR	Framework region
H	Heavy chain
HCLV	Heavy chain loss variants
HCMV	Human cytomegalovirus
HLA	Human leucocyte antigen complex
HUVEC	Human umbilical vein ECs
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Gamma interferon
Ig	Immunoglobulin
IL	Interleukin
INR	International normalized ratio
IPTG	Isopropylthiogalactoside
J	Joining region gene
Kb	Kilobase
L	Light chain
LA	Lupus anticoagulant
LB	Luria- Bertani
LDL	Low density lipoprotein
LDL-RD	Low density lipoprotein receptor deficient
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCS	Multiple cloning site
MHC	Major histocompatibility complex
mIg	Membrane-bound immunoglobulin
MW	Molecular weight
MyD88	Myeloid differentiation protein
NFκB	Nuclear factor κB
OD	Optical density
PAPS	Primary APS

PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Pulmonary embolus
PG	Phosphatidylglycerol
PL	Phospholipid
PS	Phosphatidylserine
RNA	Ribonucleic acid
SAPS	Secondary APS
scFv	Single chain fused variable region
SEC	Sample/enzyme/conjugate dilution buffer
SLE	Systemic Lupus Erythematosus
SPR	Surface plasmon resonance
TAE	Tris Acetate EDTA buffer
TBE	Tris Borate EDTA buffer
Tc	T cytotoxic cells
TE	Tris EDTA buffer
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Th	T helper cells
TIR	Toll/IL-1 receptor
TLR	Toll-like receptors
TNF	Tumour necrosis factor
UV	Ultraviolet light
V	Variable region
VBASE	Sequence directory containing the complete human Ig gene repertoire
VCAM-1	Vascular cell adhesion molecule-1
V_H	Variable region of heavy chain
V_L	Variable region of light chain
VTE	venous thromboembolism

CHAPTER ONE

INTRODUCTION

1.1 FROM IMMUNITY TO AUTOIMMUNITY

1.1.1 The normal immune response

The normal immune system protects an individual from foreign organisms or substances, called antigens (Ag), and has two functional components which act in concert. Innate immunity, the first line of defence, mounts an immediate response with disease resistance mechanisms that are not specific to a particular pathogen. This component has the advantage of speed but lacks specificity and may cause host damage. Innate immunity comprises four types of defensive barriers: anatomical, e.g. skin and mucous membranes; physiological, e.g. temperature, low pH and chemical mediators including lysosyme, interferon and complement; phagocytic e.g. neutrophils, monocytes and macrophages; and inflammatory, e.g. vasodilation, increased capillary permeability leading to an influx of phagocytes (Goldsby et al., 2000).

If these primary defences are breached specific or adaptive immunity is activated. Unlike innate immunity, adaptive immune responses are specific to a particular antigen and display four characteristic attributes: antigenic specificity; diversity; immunological memory; and self/non-self recognition. Antigenic specificity allows the immune system to recognise even single amino acid differences between antigens. A tremendous diversity is generated in the recognition molecules of the immune system such that billions of different antigens may be recognised. After initial exposure to an antigen immunological memory occurs and re-exposure to the same antigen results in a memory response which is quicker and stronger than the first conferring life long immunity to many infectious agents. Lastly, the normal functioning of the immune system is reliant upon its ability to respond only to foreign antigens as opposed to self molecules. The major cells involved in adaptive immunity are lymphocytes and antigen presenting cells (Goldsby et al., 2000).

1.1.2 Cells of the immune system

There are two major populations of lymphocytes – B lymphocytes (B cells) and T lymphocytes (T cells).

1.1.2.1 B lymphocytes

B cells comprise approximately 25% of the lymphocyte population. They mature within the bone marrow and, by the time they leave, express a unique antigen-binding receptor on their surface. This receptor is a membrane bound antibody (Ab) molecule described in detail later. Upon initial antigen encounter, a naïve B cell expressing a matching membrane bound antibody will bind to the antigen. In the presence of cytokines and B cell growth factors, the cell is activated and rapidly divides into memory B cells and plasma cells. Memory B cells have a longer life span than naïve B cells and continue to express membrane bound antibody with the same specificity as their parent B cell. Plasma cells, however, live for only a few days and do not express membrane bound antibody; instead they produce enormous amounts of the antibody in a secreted form. These secreted antibodies are the major effector molecules of humoral immunity (Morrow *et al.*, 1999; Goldsby *et al.*, 2000).

1.1.2.2 T lymphocytes

T cells also arise in the bone marrow and then, unlike B cells, migrate to the thymus gland to mature. During its maturation the T cell comes to express a unique antigen binding molecule called the T cell receptor on its membrane. Unlike B cell receptors (membrane bound antibody), T cell receptors can only recognise antigen that is bound to cell membrane proteins called major histocompatibility complex (MHC) molecules, which are polymorphic glycoproteins found on cell membranes. There are two major types of MHC molecules called class I and class II. Class I MHC molecules are expressed by nearly all nucleated cells and consist of a heavy chain linked to a small invariant protein called β2-microglobulin. Class II MHC molecules, which consist of an α and a β glycoprotein chain, are expressed only by antigen presenting cells. When a naïve T cell encounters its specific antigen combined with an MHC molecule on a cell the T cell proliferates and differentiates into memory T cells and various effector T cells (Goldsby et al., 2000).

T cells have two principal functions and are divided into two groups, T helper (Th) cells and T cytotoxic (Tc) cells. These cells can be distinguished by the presence of either CD4 or CD8 membrane glycoproteins on their surface. T cells displaying CD4 generally function as Th cells which have a variety of functions in controlling immune responses.

Once a Th cell recognises and interacts with an antigen-MHC class II molecule the cell is activated to secrete various growth factors known as cytokines. Different cytokines activate B cells, Tc cells, macrophages and various other cells that participate in the immune response and Th cells may be subdivided according to their cytokine secretion profile. Th1 cells secrete interleukin (IL)-2, IL-3 and gamma interferon (IFN)-γ which play a central part in cell mediated immunity and acute allograft rejection. Th2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-10 which enhance antibody production and humoral immunity. Cytokines also affect the differentiation of T cells into these two subsets. IFNγ and IL-12 promote Th1 differentiation and IL-4 is a potent inducer of Th2 cells. IL-10 however decreases the synthesis of Th1 cytokines. Immune responses are modulated by cytokines that affect cell proliferation, survival, differentiation, antigen presentation and trafficking (Morrow et al., 1999).

T cells displaying surface CD8 molecules generally function as Tc cells. Under the influence of Th derived cytokines a Tc cell bound to antigen presented by MHC class I molecules will proliferate and differentiate into a cytotoxic lymphocyte (CTL) which secretes fewer cytokines. They have a vital function in dealing with virus infections and cancer cells (Morrow et al., 1999).

1.1.2.3 Antigen presenting cells

Cytokines produced by Th cells are important in the activation of both the humoral and cell mediated branches of the immune system. Careful regulation of the Th cell response is ensured by virtue of the fact that these cells can only recognise antigen that is displayed in conjunction with MHC class II molecules on the surface of antigen presenting cells (APCs). These specialised cells include macrophages, B cells and dendritic cells. They express MHC class II molecules on their membranes and deliver a costimulatory signal necessary for T cell activation.

1.1.3 The role of antibodies in the immune system

Emil von Behring and Shibasaburo Kitasato in 1890 first demonstrated that a serum component could transfer immunity and in 1939 A. Tiselius and E. Kabat showed that a fraction of serum first called gamma-globulin, now immunoglobulin (Ig), was responsible for this activity (Goldsby et al., 2000). The active molecules in the Ig fraction are antibodies, the antigen binding proteins present on the surface of B cells and secreted by plasma cells.

Antibodies recognise and bind to antigens upon foreign cells or molecules that have entered the body and then either neutralize or facilitate the elimination of the foreign substance. Each antibody binds to a discrete site on the antigen. These sites are called antigenic determinants or epitopes. Once an antigen is coated with antibody, it can be eliminated in several ways. Antibody can cross-link several antigens forming clusters that are more readily ingested by phagocytic cells than a single Ab-Ag complex. Binding of antibody to antigen on a microorganism can also activate the complement system leading to lysis of the foreign organism. Toxins and viral particles coated with antibody are prevented from binding to other cells and thus neutralized.

The antigenic specificity of each B cell is determined by the membrane bound antibody molecule expressed by the cell. Antibodies can differentiate between protein antigens that differ by only a single amino acid. The generation of such antibody specificity and diversity is described in section 1.7.3. Antigen presented to B cells is degraded or processed and presented, in a MHC restricted manner, to T cells as well. Thus Th cells are generated which enhance the antibody (humoral) response made by B cells and also serve to regulate the cell mediated response in conjunction with CTLs and various phagocytic cells.

1.1.4 Antibody structure

An antibody molecule shown in Figure 1, is composed of two identical heavy (H) chains (MW 50,000 – 70,000) and two identical light (L) chains (MW 25,000). Each heavy and light chain contains intra-chain disulphide bonds and the heavy and light chains are linked to each other by disulphide bonds and noncovalent interactions.

Sequencing analysis of antibodies first became feasible with the discovery of multiple myeloma, a cancer of antibody producing plasma cells. These unregulated cancer cells escape the normal controls on their life span and continue to proliferate producing specific antibody (called myeloma protein) indefinitely. Excessive amount of myeloma derived antibody is produced compared to the amounts of antibody produced by other plasma cells.

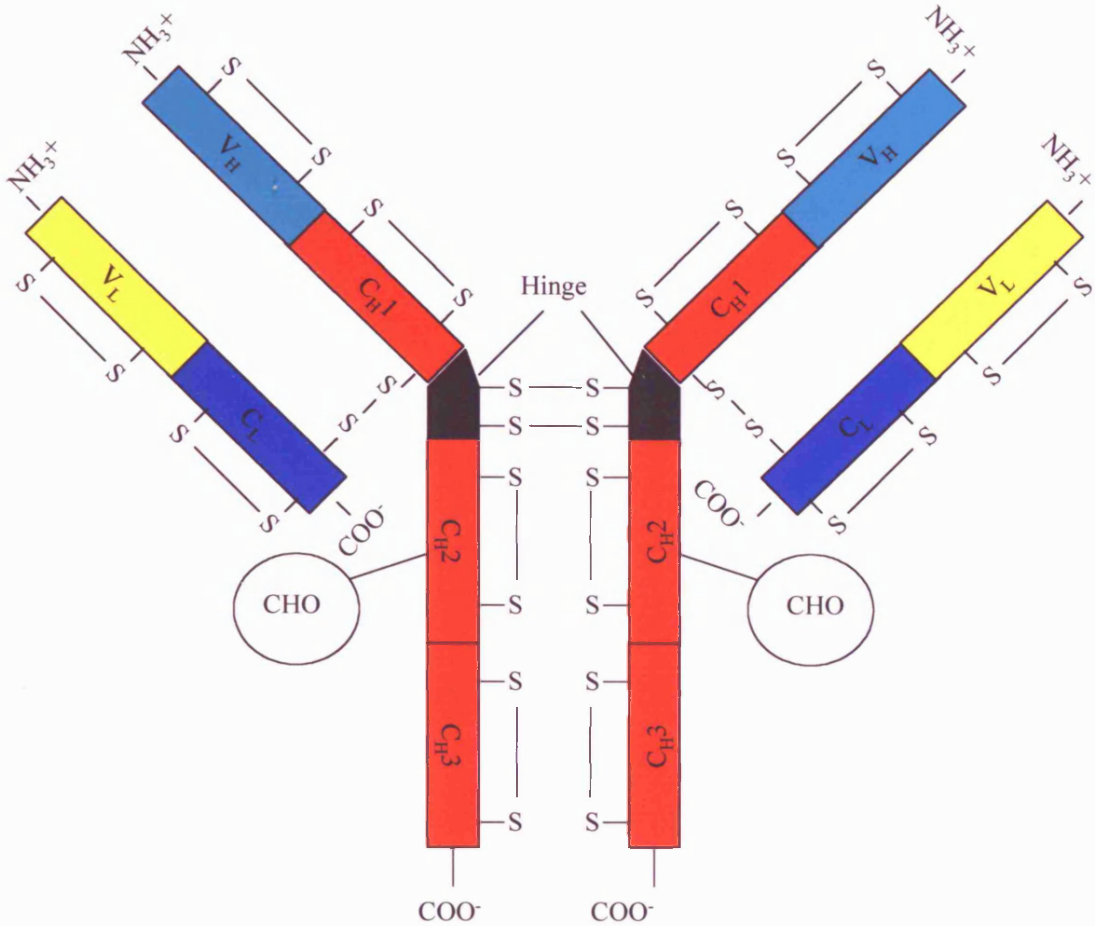


Figure 1.1 Schematic diagram of immunoglobulin structure

Abbreviations V_H – heavy chain variable region; V_L – light chain variable region; C_H - heavy chain constant region; C_L – light chain constant region; NH3+ - amino terminal; COO- - carboxyl terminus; S – sulphide group; CHO – carbohydrate moiety.

Myeloma protein produced from a particular myeloma clone is a huge source of homogenous antibody protein. Sequence analysis of myeloma proteins has revealed striking patterns within both the heavy and light chains (Wu and Kabat, 1970).

The carboxyl terminal domains of both the heavy and light chains are highly conserved and are called the constant (C) regions. These constant regions subserve effector functions, such as complement fixation, which are common to all antibodies of a given isotype. Light chain constant regions (C_L) exist in two forms (or isotypes), kappa (κ) or lambda (λ). In humans 60% of the light chains are κ (Schable and Zachau, 1993) and 40% are λ (Williams et al., 1996). In contrast, in mice 95% of the light chains are κ and only 5% λ (Kofler et al., 1992). An antibody molecule contains only one of these isotypes, never both. Amino acid differences in the λ light chains means they are further classified into subtypes. Mice have three ($\lambda1$, $\lambda2$ and $\lambda3$) and humans have four subtypes.

Heavy chain constant regions (C_H) exist in five distinct forms (μ, δ, γ, ε and α) called isotypes. The heavy chains of a given antibody determine the class of that antibody: IgM(μ), IgG(γ), IgA(α), IgD(δ) and IgE(ε). Each class can have either κ or λ light chains. As in the light chains sequence differences exist in the IgA and IgG heavy chains, hence there are two IgA subclasses ($\alpha1$ and $\alpha2$) and four IgG subclasses ($\gamma1$, $\gamma2$, $\gamma3$ and $\gamma4$).

The amino terminal domains, comprising half of each light chain and one quarter of each heavy chain, are much more variable (V) in sequence and are responsible for binding antigen. These domains are known as the heavy chain variable region (V_H) and light chain variable region (V_L). Antigen binding sites usually comprise three polypeptide loops from the V_H domain and three polypeptide loops from the V_L domain (Chothia et al., 1989). These six polypeptide loops, regions of high variability (in both sequence and length), are known as the complementarity determining regions (CDRs). The CDRs are separated by more conserved regions which perform a structural role and are hence called the framework regions (FRs) (Wu and Kabat, 1970).

1.1.5 Antigenic determinants on antibodies

Antibodies themselves may function as antigens and provoke an immune response. Antigenic determinants upon antibodies fall into three categories: isotypic, allotypic and idiotypic determinants and are all located in characteristic portions of the molecule.

Isotypic determinants are found in the constant region and determine the heavy chain class and light chain type within a species. Each isotype is encoded by a separate constant region gene. A normal individual will possess all isotypes in their bloodstream. Anti-isotype antibodies are useful in the identification of the class or sub-class of an antibody produced in an immune response either in the serum or membrane bound to a B cell.

Allotypic determinants are amino acid changes encoded by different alleles of the isotype genes that occur in some but not all members of a species. In humans allotypes exist for all four IgG subclasses, for one IgA subclass and for the κ light chain.

Antigenic determinants also reside within the amino acid sequence of the V_H and V_L domains of any given antibody. Idiotypic determinants are generated by the conformation of the V_H/V_L domains and each individual antigenic determinant of the variable region is called an idiotope. Idiotypes are usually specific for individual B cells (private idiotypes) but are sometimes shared between different B cell clones (cross-reacting, public or recurrent idiotypes).

1.1.6 Role of T helper cells in the production of antibodies

Once a naïve B cell, which has not previously encountered antigen, first encounters the antigen for which its membrane bound Ig (mIg) is specific, the B cell will bind to the antigen. Before the B cell can become activated (i.e. proliferate and differentiate) however, additional interactions with molecules on the surface of activated CD4+ Th cells and the cytokines they produce are required (Goldsby et al., 2000).

After binding of antigen by mIg on B cells the antigen is internalized by receptor mediated endocytosis and processed into peptides. This event causes levels of MHC class II molecules and co-stimulatory molecule, B7 (the ligand for T cell CD28) to be upregulated on the surface of the B cell. The internalised peptides associate with MHC class II molecules and are presented on the B cell surface to a Th cell. Once a Th cell recognises a processed peptide for which it is specific in the presence of MHC class II, the B cell surface molecule B7 simultaneously contacts its ligand on the T cell surface, CD28. This process leads to the upregulation of CD40L on the Th cell surface which binds with CD40 on the B cell. Thus B cells are stimulated to upregulate the expression of cytokine receptors so that they may interact with cytokines produced by the activated Th cell. The signals produced by these cytokine receptor interactions support B cell proliferation and can induce differentiation events which include formation of plasma and memory B cells (described earlier); class switching and affinity maturation (described later).

1.1.7 Autoantibodies and autoimmunity

Several different mechanisms of self tolerance exist to protect an individual from potentially self reactive lymphocytes. Failure of these mechanisms leads to an inappropriate response of the immune system against self components, called autoimmunity. Antibodies that bind to self-antigens are termed autoantibodies

Even in healthy individuals however, low titres of low affinity autoantibodies are part of the normal B cell repertoire (Peeva et al., 2002). These natural autoantibodies resemble the antibodies of a primary immune response in that they are primarily IgM and display polyreactive binding to a wide variety of both autoantigens and foreign antigens. Sequence analysis has revealed that they are mainly germline encoded i.e. unmutated compared to the chromosomal DNA in the B cell from which they arose (Peeva et al., 2002).

Natural autoantibodies are not unique to patients with autoimmune disease and do not appear to be pathogenic (Peeva et al., 2002). Their precise function however, is unknown and some authors have speculated that they are the precursors to pathogenic autoantibodies (Dersimonian et al., 1987). Others have suggested that these two classes of autoantibodies arise from distinct B cell populations and that pathogenic autoantibodies arise by the somatic mutation of genes that encode protective autoantibodies (Shlomchik et al., 1987).

Despite the fact that the formation of autoantibodies may be a normal physiological process, production of certain autoantibodies can cause serious damage to cells and organs sometimes with fatal consequences. Damage to the body's tissues may arise in a number of different ways. Autoantibodies may react directly with a tissue specific antigen, resulting in inflammation and tissue damage. For example, anti-glomerular basement membrane antibodies in Goodpastures syndrome cause progressive kidney failure. Alternatively, if circulating immune complexes are not cleared and large amounts remain they often deposit in tissues such as the kidney, skin, joint or nervous system and lead to tissue damaging type III hypersensitivity reactions. This damage occurs as a result of complement activation, accumulation and activation of neutrophils with the release of proteolytic enzymes and further damage. T cells are also routinely observed as infiltrates in inflammatory lesions and thought to contribute to tissue destruction, particularly in diseases such as insulin dependent diabetes mellitus and multiple sclerosis (Goldsby et al., 2000).

Autoimmune disease in humans can be divided into two broad categories; organ specific or systemic autoimmune disease. In an organ specific disease the immune response is directed to a target antigen unique to a single organ or gland so that the effects are largely localized to that organ, such as occurs in insulin dependent diabetes mellitus. In systemic autoimmune disease however, the immune response is directed to target antigens present in many tissues and organs. Systemic lupus erythematosus (SLE) and the Antiphospholipid Syndrome (APS) are both examples of systemic autoimmune disease and will be discussed later in this chapter.

1.1.8 Pathogenic autoantibodies

Autoantibodies found in SLE and APS patients tend to differ from natural autoantibodies found in healthy individuals in that they are generally IgG isotype and have a high affinity for a particular autoantigen. Certain of these autoantibodies - in particular antibodies to double stranded DNA (dsDNA) (Isenberg et al., 1997) and antiphospholipid antibodies (aPL) (Alarcon-Segovia et al., 1989) - have been implicated in disease pathogenesis. To study the sequence and structural properties of individual autoantibodies in more detail those derived from a single clone in which every cell has the same genomic DNA rearrangement and secretes the same Ig are required. These are called monoclonal antibodies.

1.1.9 Monoclonal antibodies

The preparation of homogenous antibodies with a defined specificity was achieved with the development of hybridoma technology. In 1975 a method was devised that allowed antibody secreting cells isolated from an immunised mouse to be fused with a myeloma cell (Kohler and Milstein, 1975). These hybrid cells (called hybridomas) have the immortal growth properties of the myeloma cell and the antibody producing properties of the B cell. The resultant cell lines can be maintained *in-vitro* indefinitely and will continue to secerete antibodies with a defined specificity. This method was later adapted to produce human monoclonal antibodies (Olsson and Kaplan, 1980; Teng et al., 1983).

Sequence analysis of the antigen combining sites of monoclonal Abs is a powerful tool to gain greater understanding of features at the molecular level which distinguish natural (non-pathogenic) autoantibodies found in healthy individuals from pathogenic autoantibodies found in patients with autoimmune disease. The work described in this thesis investigates the sequence and structure of aPL found in patients with APS or SLE. The features and aetiopathogenesis of these conditions are described in the next sections.

1.2 THE ANTIPHOSPHOLIPID SYNDROME (APS)

1.2.1 Definition of the APS

The APS is a multi-system disease characterised by arterial and venous thrombosis, recurrent pregnancy loss, livedo reticularis or thrombocytopenia in association with a spectrum of autoantibodies, known as aPL which are directed against phospholipids (PL) and plasma PL binding proteins (Harris *et al.*, 1983; Hughes, 1983; Asherson *et al.*, 1989; Levine and Brey, 1996). In clinical practice these antibodies are recognised by anticardiolipin (aCL) enzyme linked immunosorbent assays (ELISA) and the lupus anticoagulant (LA) assay (which detects an inhibitor of coagulation subsequently found to belong to the family of aPL). The concept of these clinical and serological disorders as a full-blown "syndrome" has only really evolved within the last twenty years. Clinical observations by earlier workers had already laid the foundation for future research.

1.2.2 The discovery of aPL and recognition of the APS

In 1906 the reagin test for the detection of a reaction between a lipoid tissue antigen and an autoantibody in syphilitic sera was developed (Wasserman et al., 1906). Initially human organs derived from patients with syphilis were used as the antigen but the test was later modified, when it was discovered that the antigen could be obtained from normal human or animal organs (Landsteiner et al., 1907). The antigen used in the majority of reagin tests was an alcoholic extract from beef heart and in 1941 this extract was found to contain a phospholipid later named cardiolipin (CL) (Pangborn, 1941; Pangborn, 1942).

During mass screening for syphilis in certain population groups in America in 1952, it was noted that large numbers of people had positive reactions to reagin tests but no clinical features of syphilis, hence the term biologically false positive serological tests for syphilis (BFP-STS) was coined (Moore and Mohr, 1952). Further testing of sub groups of these patients with chronic BFP-STS using more specific tests for syphilis (the Treponema Pallidum Immobilisation and Fluorescent Treponemal Antibody Absorption tests) gave negative results and interestingly a high incidence of autoimmune disorders, mainly SLE was found in this group of patients (Moore and Mohr, 1952).

In 1952 a circulating inhibitor of coagulation was first described in two patients with SLE and BFP-STS (Conley and Hartmann, 1952). Later work confirmed that this inhibitor was frequently associated with BFP-STS (Laurell and Nilsson, 1957). Bowie and colleagues in 1963, first described the paradoxical occurrence of thrombotic lesions in SLE patients with a circulating anticoagulant (Bowie et al., 1963) closely followed by another group in 1965 who also noted the association of a circulating anticoagulant, thrombosis and a BFP-STS in SLE patients (Alarcon-Segovia and Osmundson, 1965). Despite these early reports of association of the circulating anticoagulant with thrombotic events, the term LA was coined in 1972 (Feinstein and Rapaport, 1972). Subsequently it was confirmed that the LA assay detects aPL (Thiagarajan et al., 1980) which interfere with *in-vitro* PL dependent coagulation tests. These antibodies are not specific to patients with SLE and, paradoxically, they are associated with thrombotic events *in-vivo* (Mueh *et al.*, 1980; Boey *et al.*, 1983).

It was not until the early 1980's that the triple association of recurrent fetal losses, thrombosis and LA was made (Soulier and Boffa, 1980; Carreras *et al.*, 1981a; Carreras *et al.*, 1981b). Subsequently, an editorial in 1983 linked major cerebral disease as well as thrombosis and abortions with the LA in SLE patients and suggested the presence of a syndrome (Hughes, 1983). The subsequent development of solid phase immunoassays with

CL as the target antigen to detect aPL, initially by radioimmunoassay (Harris et al., 1983) and later by ELISA (Loizou et al., 1985) led to the recognition of the role of aCL in this disorder. Hence the term aCL syndrome was introduced in 1985 (Hughes et al., 1986) and then superseded by the APS in 1987 (Harris et al., 1987). Approximately 60% of patients with aPL have both aCL and LA whilst the remaining 40% have only one (Triplett et al., 1988). Therefore both tests are necessary in order to establish the presence of aPL.

Early studies on the assays of aCL demonstrated the need for bovine serum as a diluent and that this seemed to enhance the binding of aCL to the target antigen. In 1990 three groups independently identified a cofactor for the binding of autoimmune aCL to antigen (Galli et al., 1990; Matsuura et al., 1990; McNeil et al., 1990). This cofactor was shown to be β_2-Glycoprotein I (β_2GPI), a 50kD plasma protein that binds negatively charged PL and is described in detail later in section 1.6. In fact autoimmune aPL have been shown to be directed against a variety of other phospholipid binding serum proteins which include protein C (Oosting et al., 1993), protein S (Oosting et al., 1993) and prothrombin (PT) (Bevers et al., 1991). ß$_2$GPI however, is the most extensively studied of these protein cofactors and appears to be the most relevant clinically (McNally *et al.*, 1995; Tsutsumi *et al.*, 1996; Kandiah *et al.*, 1998).

1.2.3 Classification of the APS

APS may be diagnosed where a positive test for either aPL or LA occurs in a patient with the clinical features noted above. The presence of LA in "non-lupus patients" has been noted since the early 1980's (Hughes, 1983). It was not until the end of that decade however that the existence of a primary syndrome was recognized and classification criteria proposed in 1988 (Asherson, 1988). One year later several larger series of patients with primary APS (PAPS) were described (Alarcon-Segovia and Sanchez-Guerrero, 1989; Asherson et al., 1989; Mackworth-Young et al., 1989) and it is now clear that PAPS accounts for more than 50% of APS patients (Cervera et al., 2002). In the presence of another autoimmune rheumatic disease (ARD), most commonly SLE, the APS is considered to be secondary (SAPS).

Despite several earlier proposals for APS criteria in the 1990s (Hughes *et al.*, 1986; Harris, 1987; Alarcon-Segovia *et al.*, 1992; Alarcon-Segovia and Cabral, 1994; Wilson *et al.*,

1999) there was no attempt at a consensus until preliminary classification criteria were proposed in 1998 (Wilson et al., 1999), shown in Table 1. These criteria include two clinical outcomes vascular (arterial, venous or small vessel) thrombosis and pregnancy morbidity, either recurrent early (<10 week) losses or preterm birth due to severe placental insufficiency or pre-eclampsia. Laboratory criteria require the presence of a LA and/or moderate to high titre IgG or IgM aCL on two or more occasions at least six weeks apart. Evaluation of these criteria against APS diagnosed by experienced clinicians with a special interest in the syndrome showed acceptable sensitivity and specificity in a recent validation study (Lockshin et al., 2000). The criteria have helped to focus research but do have their limitations and were not intended to guide clinical diagnosis or treatment. In particular they make no mention of non-thrombotic manifestations of APS and do not include in the laboratory criteria any of the more recent and more specifc tests such as identification of anti-β_2GPI antibodies. These issues should be addressed in future consensus meetings.

1.2.4 Epidemiology of the APS

The prevalence of APS is unknown. aPL however are found among young, apparently healthy control subjects at a prevalence of 1.5% to 5% for both aCL and LA, which increases with age (Petri, 2000). In patients with SLE the prevalence of aPL is higher, with figures quoted of 24-39% for aCL (Alarcon-Segovia et al., 1989; Cervera et al., 1993) and 15-30% for the LA (Cervera et al., 1990; Cervera et al., 1993).

The presence of aPL, however, does not always correlate with clinical consequences. In otherwise healthy subjects, there are insufficient data to determine what percentage of those with aPL will eventually develop a manifestation of the APS (Levine et al., 2002). In contrast, the APS has been shown to develop in up to 50 to 70% of patients with SLE who are aPL positive (Alarcon-Segovia et al., 1992; Petri, 2000).

It is widely accepted that an association exists between aPL and vascular thrombosis, although there are some discrepancies in published reports due to their heterogeneity in terms of study design, enrollment criteria, associated conditions, clinical end points and laboratory tests for aPL, including isotypes measured and cutoffs taken for a positive result.

Clinical criteria

1. Vascular thrombosis: One or more clinical episodes of arterial, venous or small vessel thrombosis in any tissue or organ. Thrombosis must be confirmed by imaging or Doppler studies or histopathology, with the exception of superficial venous thrombosis. For histopathological confirmation, thrombosis should be present without significant evidence of inflammation in the vessel wall.
2. Pregnancy morbidity

a) One or more unexplained deaths of a morphologically normal fetus at or beyond the tenth week of gestation with normal fetal morphology documented by ultrasound or by direct examination of the fetus, or

b) One or more premature births of a morphologically normal neonate at or before the 34^{th} week of gestation because of severe pre-eclampsia or severe placental insufficiency, or

c) Three or more unexplained consecutive spontaneous abortions before the 10^{th} week of gestation with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

Laboratory criteria

1. Anticardiolipin antibody of IgG and/or IgM isotype in blood present in medium or high titre, on two or more occasions at least six weeks apart measured by a standard ELISA for β_2GPI dependent aCL.
2. Lupus anticoagulant present in plasma on two or more occasions at least six weeks apart detected according to the guidelines of the International Society on Thrombosis and Haemostasis.

Table 1.1 Preliminary criteria for classification of definite APS

Definite APS is considered to be present if at least one of the clinical and one of the laboratory criteria are met. Abbreviations: - ELISA – Enzyme linked immunosorbent assay; β_2GPI - β_2Glycoprotein I; aCL – anticardiolipin antibody.

A lack of standardisation of the aCL test, and the cutoff point at which a result is regarded as abnormal, is still a major problem in the study of aPL. Values for the IgG and IgM aCL tests are expressed in international units of GPLU and MPLU respectively. These units are derived from international standardised IgG and IgM calibrators (containing a defined concentration of aCL) distributed from the aPL standardisation laboratory which are then used as a standard in each ELISA (Harris and Pierangeli, 2002). Despite these efforts in standardisation which includes numerous workshops a considerable degree of interlaboratory variability still exists between laboratories, even those with extensive experience in the detection of aPL (Harris and Pierangeli, 2002).

Evidence of an association between aPL and venous thromboembolism (VTE) in patients who do not have SLE comes from various studies. A case control sub-study of 190 patients from the Physicians Health Study of healthy adult men found titres of aCL to be significantly higher in patients with VTE compared to controls although the relative risk of VTE was only statistically significant in patients with titres of aCL greater than 33GPLU (Ginsburg et al., 1992). Unfortunately the LA was not measured in this study and the methods of diagnosing VTE were not stated. A later study examined 244 unselected patients with suspected VTE and found a significant association between proven deep vein thrombosis (DVT) and pulmonary embolus (PE) with the presence of LA but not aCL (Ginsberg et al., 1995). These authors only considered an aCL ≥30GPLU as abnormal, whilst other authors and laboratories will accept ≥5GPLU as abnormal. Hence the importance of aCL ≤30GPLU was not assessed in this study. Another cross sectional study of 293 unselected patients with DVT or PE also failed to show a statistically significant association between VTE and aPL and tests for LA were not performed (Bongard et al., 1992). This study considered values of above 5 for IgG and 3 for IgM aPL to be positive, and did not exclude low positive titers of aCL (<20GPLU). The significance of low positive values is uncertain and has not been validated (Lockshin et al., 2000), which may in part explain the negative finding in Bongard`s study. Prospective studies however have shown that aPL are predictive of recurrent VTE and death (Finazzi et al., 1996; Schulman et al., 1998) as well as myocardial infarction (Vaarala *et al.*, 1995; Brey *et al.*, 2001).

Studies of all strokes have revealed conflicting results with some case control studies showing a strong association between aCL and stroke (Tuhrim et al., 1999) and recurrent stroke (Levine et al., 1992) whilst others have found no association between ischaemic stroke and aCL (Ginsburg et al., 1992). These differences may in part be explained by the use of different aCL assays and methodological differences between studies. For example, in the study of Ginsburg *et al.* (1992) cerebral infarctions occurred an average of three years after samples for aCL were obtained and assays were performed after an average of eight years of frozen storage which may have affected the chance of obtaining a positive aCL. A large prospective nested case control study has clearly demonstrated an association of aPL, particularly β_2GPI dependent aPL, with stroke (Brey et al., 2001).

The strongest association of aPL with pregnancy morbidity is with late fetal loss beyond 10 weeks or more of gestation (Lockshin et al., 1985; Oshiro et al., 1996). In contrast, fetal losses due to other causes (e.g. chromosomal abnormalities) usually occur within the first nine weeks of pregnancy (Levine et al., 2002).

1.2.5 Risk factors for thrombosis in aPL positive patients

Various studies have tried to assess which factors in aPL positive patients are associated with an increased risk for a thrombosis. A four year, randomised, multicentre, prospective study of unselected patients, comparing different durations of oral anticoagulation, measured IgG aCL six months after a first or second episode of VTE (Schulman et al., 1998). Among 412 patients with a first episode of VTE the risk of recurrence was found to be 29% in patients with aPL and 14% in those without aPL, a statistically significant difference. This risk was increased during the first six months after cessation of anticoagulation and with moderate or high titers of IgG aCL, >35GPLU although this association was not described statistically (Schulman et al., 1998). A weakness of this study is that the presence and titer of aPL at the time or prior to VTE was not examined hence it could be argued that the aPL may be an epiphenomenon post VTE. This explanation is unlikely however because similar results have been obtained from prospective studies which measured aPL before or at the time of VTE. One four year multicentre study, prospectively followed 360 consecutive unselected cases of aPL positive patients (Finazzi et al., 1996). In this study the total incidence of thrombosis was 2.5% per year and a history of thrombosis and IgG aCL titer > 40GPLU were the most statistically significant predictors for thrombosis. Other prospective studies have also demonstrated a statistically significant association between an elevated level of IgG aCL and thrombosis (Alarcon-Segovia et al., 1989), pregnancy morbidity (Lynch et al., 1994) and myocardial infarction (Vaarala et al., 1995).

A systematic review and meta-analysis of 26 articles examining the association between aPL and VTE found that SLE patients with the LA compared to those lacking LA had approximately a six fold greater risk for VTE (Wahl et al., 1997). The risk of VTE in patients with SLE who were aCL positive was approximately twice that of patients lacking aCL (Wahl et al., 1997). There are methodological limitations to this analysis since it combined studies of differing quality and design. Nonetheless, their finding of LA being the strongest risk factor for VTE in SLE patients has been confirmed in a retrospective study (Horbach et al., 1996). In fact a recent systematic review of 25 studies on more than 7000 patients and controls found that LA were statistically stronger risk factors for thrombosis than aCL regardless of the site (arterial or venous) or type (first event or recurrence) of thrombosis and the presence of SLE (Galli *et al.*, 2003b).

Of the various cofactors that have been identified, antibodies against β_2GPI have been shown in several case control studies to have a statistically significant association with VTE in patients with either PAPS or SLE/APS (Martinuzzo et al., 1995; McNally et al., 1995) and have been reported to be more specific than aCL in detecting manifestations of the APS (Roubey et al., 1996). A recent systematic review of 28 mainly retrospective studies found a statistically significant association between anti-β_2GPI antibodies and thrombosis in 34 (57%) of 60 associations (mostly venous thrombosis) examined, particularly with IgG and IgA antibodies (Galli *et al.*, 2003a). Overall this association was not as strong as for the LA and thrombosis. Laboratory studies have also shown that binding to β_2GPI is not an absolute requirement in determining the pathogenicity of aPL as human monoclonal IgG aPL that bind PL in the absence of β_2GPI have been isolated and shown to be highly pathogenic *in-vivo* (Ikematsu *et al.*, 1998; di Simone *et al.*, 2000).

1.2.6 Clinical Manifestations of the APS

1.2.6.1 Thrombotic manifestations

Thrombosis within the venous or arterial circulation is a major clinical feature of this syndrome, see Table 1.2. Any size of vessel may be affected. Venous thrombosis, especially deep venous thrombosis of the legs, is the most common manifestation, occurring in 29-55% of APS patients during an average follow up of less than six years (Asherson *et al.*, 1989; Alarcon-Segovia *et al.*, 1992; Vianna *et al.*, 1994). PE are also frequent occurring in up to half of these patients (Asherson *et al.*, 1989; Alarcon-Segovia *et al.*, 1992; Vianna *et al.*, 1994). Arterial thromboses are less common and most frequently affect the brain with transient ischaemic attacks and strokes accounting for 50% of arterial occlusions (Asherson et al., 1989). A further 23% of arterial occlusions occur in coronary arteries with the remainder involving diverse areas including subclavian, renal, retinal and pedal arteries (Asherson et al., 1989). Arterial emboli, particularly from mitral or aortic valve vegetations found in 4% of APS patients (Vianna et al., 1994) may also lead to cerebral events. Thrombotic microangiopathy may occur as a result of microvascular involvement either acutely, resulting in a clinical picture similar to the haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura, or as a more chronic process leading to a slow progressive loss of organ function.

Other prominent manifestations of the APS include thrombocytopenia (40-50% of patients), haemolytic anaemia (14-23% patients) and livedo reticularis (11-22% patients) (Asherson *et al.*, 1989; Alarcon-Segovia *et al.*, 1992; Vianna *et al.*, 1994).

1.2.6.2 Obstetric manifestations

The other major clinical feature of the APS is pregnancy morbidity, shown in Table 1.2, and the recent classification criteria (described in section 1.2.3) now recognise that other adverse pregnancy outcomes as well as late fetal loss, may also occur in pregnant women who are aPL positive (Wilson et al., 1999). These adverse outcomes include premature delivery due to pregnancy associated hypertensive disease and uteroplacental insufficiency (Branch et al., 1992) and recurrent (three or more) consecutive spontaneous abortions before the 10th week of gestation with other medical causes excluded (Kutteh, 1996; Rai et al., 1997).

Organ system	Clinical manifestations of the APS
Cardiac	Angina, myocardial infarction, cardiac valvular vegetations, valvular abnormalities, intracardiac thrombi, nonbacterial thrombotic endocarditis, peripheral embolisation or atherosclerosis
Cutaneous	Livedo reticularis, superficial thrombophlebitis, splinter haemorrhages, leg ulcers, distal cutaneous ischaemia, infarcts of the skin,
Endocrine	Adrenal infarction, adrenal failure, testicular infarction, prostate infarction, pituitary gland necrosis or failure
Gastrointestinal	Budd-Chiari syndrome, hepatic infarction, intestinal infarction, splenic infarction, oesophageal perforation, ischaemic colitis, pancreatitis or ascites
Haematologic	Thrombocytopenia, haemolytic anaemia or haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura
Limb vessels	Deep vein thrombosis, claudication, ischaemia, gangrene
Miscellaneous	Perforation of the nasal septum or avascular necrosis
Neurologic	Transient ischaemic attack, cerebrovascular accident, chorea, seizures, mulit-infarct dementia, transverse myelitis, encephalopathy, migraines, pseudotumour cerebri,cerebral sinus thrombosis, mononeuritis multiplex or amaurosis fugax
Obstetrical	Pregnancy loss, intrauterine growth retardation, HELLP syndrome (haemolysis, elevated liver enzymes and a low platelet count in association with preeclampsia), oligohydramnios, uteroplacental insufficiency or preeclampsia
Ophthalmologic	Retinal artery or vein thrombosis
Pulmonary	Pulmonary emboli, pulmonary hypertension, pulmonary arterial thrombosis or alveolar haemorrhage
Renal	Renal vein thrombosis, thrombosis of the renal artery, renal infarction, hypetension, acute renal failure, chronic renal failure, proteinuria, haematuria or the nephrotic syndrome

Table 1.2 Clinical manifestations of the antiphospholipid syndrome

1.2.6.3 The catastrophic APS

Less than 1% of APS patients present with an acute and devastating syndrome characterised by multiple simultaneous vascular occlusions throughout the body, often resulting in death (Erkan et al., 2003). This syndrome, called the catastrophic APS (CAPS) differs from classic APS in several ways. Vascular involvement of large vessels is less common in patients with CAPS who tend to present with severe multiple organ dysfunction characterised by rapid, diffuse small vessel ischaemia and thromboses predominantly affecting the parenchymal organs (Erkan et al., 2003). The clinical characteristics and outcomes of 130 reported CAPS patients have been reviewed in two different series (Asherson et al., 1998; Asherson et al., 2001) and an international registry for these patients has now been created (Erkan et al., 2003). Based on these series, 63 of 130 CAPS patients (43%) died at the time of the reported event. Older age and a higher number of involved organs particularly the spleen, heart and intestine were associated with mortality (Erkan et al., 2003).

1.2.6.4 Non-thrombotic manifestations of aPL

Although thrombosis underlies many complications of the APS some manifestations especially those affecting the nervous system are difficult to explain solely on the basis of a hypercoagulable state. Support for a direct antibody mediated interaction comes from both animal and human data. Animal work has shown aPL to bind directly to cat brain (Kent et al., 1997), whilst in snails aPL reduce a GABA receptor mediated current in snail neurons (Liou et al., 1994). Thus aPL may directly and reversibly lower seizure threshold by binding to brain PL. In APS patients with reversible chorea it has been postulated that the underlying mechanism may be related to striatal binding of aPL rather than aPL associated striatal ischaemia. Two case reports of aPL associated chorea using serial PET scans have shown transient hypermetabolism in the contralateral basal ganglia of both patients studied (Furie et al., 1994; Sunden-Cullberg et al., 1998) suggesting an underlying excitatory rather than ischaemic pathophysiologic mechanism.

Pregnancy morbidity in APS patients may result from poor placental perfusion (De Wolf et al., 1982) due to localised thrombosis, possibly by interference with trophoblastic annexin V that is mediated by aPL (Rand et al., 1997). It has also been proposed however that aPL may also impair trophoblast invasion and hormone production, thereby promoting not only pre-embryonic and embryonic loss but also fetal loss and uteroplacental insufficiency (di Simone et al., 2000).

1.2.7 Is it important to differentiate between primary and secondary APS?

The question of whether the presence of SLE modifies in any way the clinical or serological expression of the APS was first addressed by Vianna and colleagues (Vianna et al., 1994). They compared 56 SLE/APS patients with 58 PAPS patients and found similar clinical and laboratory profiles in both groups, with the exception of autoimmune haemolytic anaemia, endocardial valve disease, neutropenia and low C4 levels which were all more common in SLE/APS patients. More recently a pan-European study of 1,000 unselected patients with definite APS, according to the preliminary classification criteria (Wilson et al., 1999), analysed the prevalence and characteristics of the main clinical and immunological manifestations of both PAPS and SAPS at disease onset and during its evolution to define patterns of disease expression (Cervera et al., 2002). APS was primary in 53.1% of the patients and associated with SLE in 36.2%. A wide variety of thrombotic manifestations were recorded during the evolution of the disease in vessels of almost all organ systems. Patients with APS associated with SLE had more episodes of arthritis, livedo reticularis, thrombocytopenia and leucopenia. There were also differences related to sex as all female patients had a higher frequency of arthritis, livedo reticularis, and migraine. Male patients had a higher frequency of myocardial infarction, epilepsy, and arterial thrombosis in the lower legs and feet. Patients in whom APS occurred before the age of 15 had more episodes of chorea and jugular vein thrombosis whilst those with disease onset after the age of 50 had a higher frequency of stroke and angina pectoris, but a lower frequency of livedo reticularis, than the remaining patients (Cervera et al., 2002).

This study is important because it is the largest cohort of patients described to date with definite APS identified by the current classification criteria and thus truly representative of what are currently accepted as patients with the syndrome. It allows a more precise estimate of the prevalence of both major and minor features compared to previous studies, as well as the analysis of the pattern of disease expression in specific APS groups. Factors independent of coexistent SLE were found to modify APS expression and define specific subsets of the disease. The fascinating question of whether the APS patients with different patterns of disease expression also have a different prognosis will be assessed during the next ten years of follow up of this cohort.

1.2.8 Prognosis in APS

Elevated titers of aPL six months after an episode of VTE, in unselected patients, increases the risk of recurrence to 29% and four year mortality to 15%, compared with recurrence rates of 14% and 6% mortality in aPL negative patients, regardless of the duration of warfarin treatment (Schulman et al., 1998). Other studies have also shown that APS carries a heavy disease burden with recurrence rates of up to 29% for thrombosis and mortality of up to 10% over a follow-up period of ten years, despite the currently best available treatment of life long anticoagulation (Shah et al., 1998).

1.2.9 Management of APS patients

Treatment of the APS falls into four main categories: - prophylaxis; prevention of further thrombosis of large vessels; treatment of acute thrombotic microangiopathy; and management of pregnancy in aPL positive patients.

1.2.9.1 Prophylaxis

Aspirin (325mg/day) was not protective against the occurrence of DVT and PE in 190 healthy male physicians, followed for 60 months, found to be aCL positive (Ginsburg et al., 1992). In contrast aspirin (81 – 325mg) did reduce the incidence of subsequent thrombosis in a subgroup of 65 APS patients with pregnancy morbidity and no previous thrombosis, followed for on average eight years (Erkan et al., 2001). Hydroxychloroquine may be protective against thrombosis in SLE patients with APS (Petri, 1996). Any other risk factors for thrombosis should be identified and treated such as defects in coagulation factors and homocystinaemia. In addition, modification of any secondary risk factors for athersclerosis (AS) such as smoking, hypercholesterolaemia, hypertension, and diabetes is advisable given the putative role of aPL in AS (see section 1.5.4). Hormone replacement therapy should be avoided in view of its prothrombotic potential.

1.2.9.2 Treatment after a thrombotic event

Anticoagulation after a thrombotic event is the only treatment which has been shown to reduce the rate of further thrombosis in both retrospective (Derksen et al., 1993) and prospective studies (Rosove and Brewer, 1992; Khamashta *et al.*, 1995). The exact level of anticoagulation however remains less clear. A retrospective study of 19 primary and secondary APS patients found that patients treated with oral anticoagulants at an international normalized ratio (INR) of 2.5 – 4.0 had a 100% probability of survival free from VTE at 8 years of follow up (Derksen et al., 1993). By comparison among patients whose anticoagulation was stopped the rate of recurrence was 50% at two years and 78% at eight years of follow up (Derksen et al., 1993). In two larger series which were both retrospective the level of protection against arterial and VTE correlated directly with the intensity of anticoagulation (Rosove and Brewer, 1992; Khamashta *et al.*, 1995). One study of 70 primary and secondary APS patients found recurrent events in 53% of patients and that the site of the first event (arterial or venous) tended to predict the site of subsequent events (Rosove and Brewer, 1992). In this study there were fewer recurrent thrombotic events in patients treated with warfarin as opposed to those patients who received no treatment or aspirin alone. In the warfarin treated patients there were nine thrombotic recurrences in seven patients, six of which occurred during low intensity treatment (INR < 2.0) and three during intermediate intensity treatment (INR 2.1 – 2.9). There were no recurrent thrombotic events in patients who received high intensity warfarin treatment (INR ≥ 3.0) during 110.2 patient years of follow up (Rosove and Brewer, 1992). Similar results were found in a series of 147 APS patients (Khamashta et al., 1995). In both of these studies aspirin alone was ineffective in reducing the rate of recurrent thrombosis (Rosove and Brewer, 1992; Khamashta *et al.*, 1995).

Results from these retrospective studies (Rosove and Brewer, 1992; Khamashta *et al.*, 1995) showed a clear benefit of anticoagulation in preventing recurrent thrombosis which was greater at a higher intensity of anticoagulation (INR ≥ 3.0). In view of the retrospective nature of these studies however, and conflicting results from other studies in which intermediate intensity warfarin has appeared to be effective in suppressing recurrent thrombosis (Ginsberg et al., 1995), the benefits of high intensity coagulation remain unclear given its higher risk of haemorrhagic complications.

A recent randomized double blind trial has attempted to address the question of whether APS patients should be treated with high versus medium intensity warfarin (Crowther et al., 2003). This study recruited 114 primary and secondary APS patients and followed them for a mean of 2.7 years. There were six recurrent thrombotic events among the 56 patients in the high intensity warfarin group (INR 3.1 – 4.0) compared with two in 58 patients in the medium intensity warfarin group (INR 2.0 – 3.0). The incidence of major bleeding was similar in both groups. Therefore the conclusion from this study was that high intensity warfarin was not superior to moderate intensity warfarin for thromboprophylaxis in APS patients with prior thrombotic events. A closer inspection of the results however reveals that although the average INR values in the moderate and high intensity warfarin groups were within their target range 2.3 and 3.3 respectively, the INR was kept within its target range 71% of the time in the moderate intensity group and in only 40% of the time in the high intensity group. In this latter group the target INR was sub-therapeutic (i.e. < 3.0) 43% of the time. The two recurrent thrombotic events in the moderate intensity group occurred at INRs of 1.6 and 2.8 and, of those six events which took place in the high intensity group, one occurred in a patient who had discontinued warfarin 137 days previously and the remaining five at INRs of 3.1, 1.0, 0.9, 1.9, and 3.9. Therefore of the nine recurrent thrombotic events seen in this study only two occurred at an INR ≥ 3.1 and seven occurred in patients with an INR of ≤ 2.8. Therefore I believe that further studies are required with tighter control of the INR in different intensity of treatment warfarin groups to decide whether patients should receive high or medium intensity warfarin treatment.

1.2.9.3 Management of pregnancy in aPL positive patients

Studies of treatment of pregnancy morbidity in patients with APS have mainly centred upon the prevention of fetal loss. Early studies using high doses of Prednisolone ($\geq$ 60mg daily) reported improved fetal survival (Lubbe *et al.*, 1983; Kwak *et al.*, 1994). Later studies however have demonstrated that aspirin and heparin gave equivalent fetal outcomes when compared with aspirin and steroids with significantly less fetal morbidity (Cowchock et al., 1992). Therefore the use of steroids in APS pregnancies has been abandoned. Two prospective randomised studies have shown that heparin plus low dose aspirin provides a significantly better outcome than low dose aspirin alone (Kutteh, 1996; Rai et al., 1997). A more recent study however failed to demonstrate any additional efficacy with low molecular weight heparin plus aspirin compared to aspirin alone in APS patients with recurrent miscarriage and no thrombotic events (Farquharson et al., 2002). In practice, heparin is offered in addition to aspirin to APS patients with no thrombotic events who have had one or more second trimester loss or three or more first trimester losses.

In APS patients with a previous history of thromboembolism it is common practice to administer low molecular weight heparin throughout pregnancy as well as aspirin (Lakasing et al., 2000). If a thrombotic event occurs during an APS pregnancy despite heparin thromboprophylaxis, or in patients with a history of previous cerebrovascular thromboses, the risk of recurrence is sufficiently high to consider antenatal administration of warfarin (Hunt et al., 1998). In practice the use of warfarin is avoided in the first trimester of pregnancy because of its potential for teratogenicity and is switched to heparin two weeks prior to planned delivery to allow clearance of warfarin by both mother and fetus.

Immunosuppression with azathioprine, intravenous Ig, plasma exchange and IL-3 therapy has all been tried in APS pregnancies (reviewed in (Lakasing *et al.*, 2000)). Due to the variable course of the disease and small numbers of patients involved however any potential benefits remain unproven.

1.2.9.4 Treatment of catastrophic APS

Early diagnosis and aggressive treatment are crucial in the management of CAPS patients. A high index of clinical suspicion and careful investigation are required. A recent review of data from the CAPS registry recommends that as soon as the diagnosis of CAPS is suspected, anticoagulation and corticosteroids should be commenced (first line treatment). Furthermore, intensive care therapy, haemodialysis for renal failure, mechanical ventilation for respiratory failure or inotropic drugs for cardiogenic shock also play an important role in the management of CAPS patients (Erkan et al., 2003).

Treatment or elimination of possible precipitating factors - such as infections, tissue necrosis, drugs (mostly oral contraceptives), or surgical procedures - is also very important. Medical management is centered on anticoagulation (usually intravenous heparin followed by a warfarin derivative), corticosteroids, plasmapheresis and intravenous gammaglobulins are the most commonly prescribed medications for CAPS patients and seem to be the most useful (Erkan et al., 2003). A variety of other management options have been reported for CAPS patients where either no benefit was found e.g. with the addition of cyclophosphamide or they were only used in single cases e.g. defibrotide, hence their benefit is doubtful (Erkan et al., 2003).

1.3 SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is a complex clinical syndrome of multifactorial aetiology, which I shall describe in brief since this thesis is primarily about aPL and the APS. SLE is characterised by widespread inflammation, most commonly affecting women during the child bearing years. It is characterised by periods of relative quiescence and periods of exacerbations, which may involve any organ or system in various combinations (Isenberg and Horsfall, 1998). Because of its multisystem involvement and protean manifestations, the diagnosis of SLE may be difficult. The American College of Rheumatology have published criteria for the classification of patients as having SLE (Tan et al., 1982) which were revised in 1997 (Hochberg, 1997) to include "positive finding of aPL" in the immunological disorder criterion, shown in Table 1.3. Although these criteria were designed primarily as a research rather than diagnostic tool they are useful in evaluating individual patients and it is widely accepted that if a patient has, at any time in their medical history at least four of the 11 criteria documented, the diagnosis of SLE can be made.

1.3.1 Aetiology of SLE

The precise aetiology of SLE remains unclear. However the cardinal features of the abnormal immune response: - defects in B cell hyperactivity, contributing to hypergammaglobulinaemia and pathogenic autoantibody production; impaired cell mediated immunity; a predominance of Th2 cytokines; dysfunction of apoptosis or in the clearance of apoptotic material; and upregulation of adhesion molecules are well described, for review see (Mason and Isenberg, 1998). The marked diversity seen in the immunological abnormalities undoubtedly has a genetic predisposition and in general terms SLE occurs in an individual when the appropriate stimulus is delivered to a genetically and probably hormonally susceptible person.

Item	Definition
Malar rash	Fixed erythema, flat or raised, over the malar eminences, sparing the nasolabial folds
Discoid rash	Erythematosus raised patches with adherent keratotic scaling and follicular plugging: atrophic scarring may occur in older lesions
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless observed by a physician
Nonerosive arthritis	Involving 2 or more peripheral joints, charatcerised by tenderness, swelling or effusion
Pleuritis or pericarditis	a. Pleuritis - convinving history of pleuritic pain or rub heard by a physician or evidence of pleural effusion OR b. Pericarditis - documented by electrocardiogram or rub or evidence of pericardial effusion
Renal disorder	a. Persistent proteinuria > 0.5gm per day or >3+ if quantitation not performed OR b. Cellular casts - may be red cell, haemoglobin, granular, tubular or mixed
Neurologic disorder	a. Seizures OR b. Psychosis - in the absence of offending drugs or known metabolic derangement e.g. uraemia, ketoacidosis or electrolyte imbalance
Haematologic disorder	a. Haemolytic anaemia with reticulocytosis OR b. Leucopenia- $<4{,}000/mm^3$ on ≥ 2 occasions OR c. Lymphopenia- $<1{,}500/mm^3$ on ≥ 2 occasions OR d. Thrombocytopenia- $<100{,}000\ mm^3$ in the absence of offending drugs
Immunologic disorder	a. Anti-DNA: antibody to native DNA in abnormal titre OR b. Anti-Sm: presence of antibody to Sm nuclear antigen OR c. Positive finding of aPL based on : 1) an abnormal serum level of IgG or IgM aCL, 2) a positive test result for LA using a standard method, or 3) a false positive test result for syphilis sustained for at least 6 months and confirmed by *Treponema pallidum* immobilisation or fluorescent treponemal antibody absorption test
Anti-nuclear antibody	An abnormal titre of ANA by immunofluorescence or an equivalent assay at any point in time in the absence of drug

Table 1.3 1997 update of the 1982 American College of Rheumatology classification criteria for systemic lupus erythematosus

Adapted from Tan *et al.* (1982) and Hochberg (1997).

1.3.2 Epidemiology and natural history of SLE

SLE is found worldwide with racial and geographic variation; hence it is much more common among black females in the United Kingdom (UK), the West Indies and the United States of America (Morrow et al., 1999). A UK study has recorded prevalence rates of 36.2, 90.6 and 206 per 100,000 among women of Caucasian, Asian and Afro-Caribbean origin respectively (Johnson et al., 1995).

Women are affected with SLE between 10 – 20 times more frequently than men and the overwhelming majority of patients will develop their disease between the ages of 15 and 40 years. It is now recognised that in 10 – 15% of patients the disease will begin after the age of 50 and in this subgroup the female to male ratio falls to around 4:1 (Morrow et al., 1999).

The prognosis of SLE has improved dramatically in the last 40 years with survival rates quoted of ~90% at 10 years after diagnosis and ~70% at 20 years (Rus and Hochberg, 2002). Deaths early in the course of SLE are usually attributed to active disease and infections, but deaths that occur later in the disease course are often due to atherosclerotic vascular disease (Esdaile et al., 2001; Roman et al., 2003). Despite the marked improvement in survival SLE remains a disease with the potential to cause considerable morbidity and increased mortality in a young age group.

1.3.3 Clinical features

The musculoskeletal system and skin are organ systems most frequently affected. Non-specific features such as severe fatigue, fever, anorexia, weight loss and lymphadenopathy are commonly found in the presence of active disease. Arthralgia occurs in about 90% of SLE patients and is usually polyarticular, symmetrical, episodic and may be flitting in nature. Clinical evidence of erosive arthritis however is only found in 5% of patients. Similarly although myalgia is reported in up to 50% of patients a true myositis is found in only 5% of these patients (Isenberg and Horsfall, 1998).

A variety of skin lesions are seen in SLE patients including alopecia , the classic butterfly rash, maculopapular and discoid lesions, splinter haemorrhages, dilated nailfold capillaries, bullous lesions, angioneurotic oedema as well as buccal and nasal ulceration are all widely recognised (Isenberg and Horsfall, 1998). Many of these skin lesions are photosensitive thus sun protection forms an important part of management of SLE. A variant of SLE known as subacute cutaneous lupus erythematosus is well recognised and associated with anti-Ro antibodies. Immunoglobulin deposition at the dermal/epidermal junction may be found in lesional and non-lesional areas of skin.

A normochromic, normocytic anaemia is present in up to 70% of SLE patients and a Coombs' positive haemolytic anaemia in 10% of all patients. Leucopenia and lymphopenia are the most frequent abnormalities of the white blood cell count found in SLE patients. Thrombocytopenia may present in one of three ways; a chronic form which is rarely associated with bleeding episodes; acutely where the fall in the platelet count may be both dramatic and life threatening; or as what initially appears to be an idiopathic thrombocytopenia which is treated but other manifestations of SLE later develop (Morrow et al., 1999).

Despite the high incidence of abnormal pulmonary function tests reported in 85% of SLE patients the commonest clinical features of pulmonary disease, dyspnoea and pain due to pleurisy (often associated with an effusion) are seen in only about 40% of patients. Parenchymal involvement due to SLE is found less commonly in 18% of patients and true SLE pneumonitis is rare in less than 2% of patients (Morrow et al., 1999). Many pulmonary lesions are the result of concomitant infection, congestive cardiac failure or drug toxicity. The presence of pulmonary thromboembolic disease should prompt the clinician to search for the presence of aPL. These antibodies should also be sought in the small numbers of SLE patients who develop pulmonary hypertension (D'Cruz et al., 2002).

The pericardium, myocardium and endocardium may all be affected by SLE. Electrocardiographic and echocardiographic studies have all shown that involvement of these structures is more common than is suspected on clinical examination. Although, up to 30% of SLE patients approximately may have clinical evidence of pericardial disease, large pericardial effusions are rare. Clinical myocarditis occurs in up to 15% of patients but valvular lesions rarely cause significant lesions (Morrow et al., 1999).

Significant renal disease will develop in approximately 30% of SLE patients. Clinical symptoms suggesting renal involvement rarely become evident until substantial damage has been done. Thus careful monitoring of the blood pressure for hypertension, the urine for protein, red cells or casts, and the plasma for raised plasma creatinine and urea levels is mandatory. The World Health Organisation has classified SLE renal disease into five major categories according to information derived from renal biopsy (Isenberg and Horsfall, 1998). This classification however is constantly being revised and the exact role of renal biopsy in SLE remains a matter of debate.

Virtually every feature of central nervous system disease has been described in SLE patients, from the common migraine through to less common features such as major psychotic episodes, grand mal seizures and rarely movement disorders. The neurological manifestations of SLE may be peripheral and psychiatric as well as central. In fact up to 70% of SLE patients have been reported to suffer from some sort of neurological or psychiatric abnormality (Isenberg and Horsfall, 1998).

Vascular lesions particularly Raynaud`s phenomenon which occurs in a third of patients, as well as cutaneous vasculitis, ulcers and digital gangrene are also well recognised in SLE patients (Isenberg and Horsfall, 1998). Abdominal pain is found in 10 – 20% of the patients, the causes of which range from mild non-specific gastroenteritis to life threatening mesenteric vasculitis. Hepatomegaly and/or persistent liver function test abnormalities as well as splenomegaly are found in up to 10% of patients but pancreatitis is less common (Morrow et al., 1999).

There has been a marked improvement in the five year survival of this disease over the last 20 years but it still remains a disabling and potentially fatal disease that tends to affect women in the prime years of their lives. Current treatments available are limited, especially in view of potentially severe side effects and more targeted therapies are needed. Therefore much research has tried to gain a greater understanding of the pathogenesis of this disease and autoantibodies are among the main areas of interest.

1.3.4 Autoantibodies in SLE

A variety of autoantibodies are found in SLE patients, see Table 1.4. Given the vast number of targets available within the cell (around 2000) however, the diversity of autoantibodies produced in lupus patients is actually rather restricted. These autoantibodies may be directed against: cell nucleus components e.g. antinuclear antibodies (ANA) and anti-dsDNA antibodies; cytoplasmic antigens e.g. heat shock proteins; cell membranes e.g. anti-platelet antibodies and aPL; and serum components e.g. C1q.

Screening for ANA is an important primary step in the diagnosis of lupus. Detection of specific autoantibodies is then required by more specific tests and even then not all autoantibodies can identify a specific disorder (Smeenk et al., 1985). Consideration of autoantibody profiles however increase diagnostic predictive value without loss of specificity or sensitivity for example in identifying SLE (by the presence of antibodies to dsDNA and/or Sm) from other ARD, such as Sjogren's syndrome (associated with anti-Ro and/or anti-La) or systemic sclerosis (anticentromere and/or Scl 70 antibodies) (Juby et al., 1991). The same autoantibody profiles may also be used to identify disease subsets in SLE patients. Thus patients with antibodies to dsDNA and/or Sm have a significant increase in malar rash, hypocomplentaemia, renal and haematological involvement whilst patients with anti-Ro and/or anti-La antibodies have a worse lupus rash and photosensitivity (Thompson et al., 1993). aPL are found in 20-50% of lupus patients and LA in 15-35% and are associated with the manifestations of the APS. Therefore more diagnostic and prognostic power is conferred when autoantibody profiles are considered as a whole in each patient.

1.3.5 Anti-dsDNA antibodies

Of the disease specific antibodies in patients with SLE those targeting dsDNA carry the most diagnostic power and clinical application. Between 60-83% of lupus patients are found to have anti-dsDNA antibodies and in some patients the titre of these antibodies is an excellent measure of disease activity (Hahn, 1998). These antibodies are almost specific to patients with SLE and are rarely seen in patients with other diseases or in healthy people (Isenberg et al., 1985). Levels of IgG anti-dsDNA antibodies, in particular, are closely correlated with the degree of renal damage in lupus nephritis and have been shown to deposit in the kidneys of SLE patients (Hahn, 1998). Only a subset of anti-dsDNA antibodies appears to be pathogenic however, because not all patients with high serum

Antibody specificity	Literature (%)	CFR (%)
ANA	>90	95
dsDNA	40-90	61
ssDNA	Up to 70	-
Histone	30-80	-
Sm	5-30	10
RNP	20-35	22
Ro	30-40	33
La	10-15	12
CL	20-50	23(IgG) 10(IgM)
LA	10-20	13
Fc IgG (RF)	25	23
Thyroid Ags	Up to 35	11

Table 1.4 Approximate prevalence of antibodies detectable in the serum of SLE patients

The literature column refers to an approximate range from several published studies. The CFR column is based on the first 388 patients with SLE under long term follow up at the Centre for Rheumatology (CFR), Middlesex Hospital, London.

ANA (substrate rat liver) ≥ 1:80 positive result. RF ≥ 1:80 positive result.

Abbreviations: ANA – Anti-nuclear antibody; ds – double stranded; ss – single stranded; CL – cardiolipin; LA – lupus anticoagulant; RF – rheumatoid factor.

Adapted from Table 4.10, Morrow *et al.* (1999) with personal communication from Professor D. Isenberg.

levels of these antibodies have high disease activity. Similarly, not all anti-dsDNA antibodies tested in experimental models can cause tissue damage (Ehrenstein et al., 1995). Sequence analysis of monoclonal anti-dsDNA antibodies has proved invaluable in determining the molecular features which distinguish potentially pathogenic antibodies from those which are less likely to be pathogenic.

1.3.6 Management of SLE

All SLE patients need education, counseling and support due to the complexity and unpredictability of the disease process. General measures include advice to minimise any factors that may precipitate a disease flare such as: sun exposure; undue stress; and infection where possible. Preventative measures to avoid infection include influenza and pneumococcal vaccination; live vaccines however should be avoided in patients taking immunosuppression or >10mg prednisolone/day. Adequate contraception is vital particularly when lupus is active and the patient is taking cytotoxic drugs. Since cardiovascular disease is a major cause of long term morbidity and mortality among SLE patients (Esdaile et al., 2001; Roman et al., 2003), strategies to identify and treat cardiovascular risk factors are also very important.

The degree of further treatment required in each SLE patient depends upon the range of disease manifestations present. SLE patients with mild disease (fatigue, fever, rash, arthralgia and mild serositis) can usually be managed with a combination of topical high factor sunscreens, glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs) and antimalarials such as hydroxychloroquine or mepacrine (Morrow et al., 1999).

Oral corticosteroids are required when NSAIDs and antimalarials have failed to control the disease or there is major organ involvement. Low dose prednisolone (<0.5mg/kg/day) is often sufficient to gain disease control. Severe organ involvement, such as nephritis or vasculitis, requires systemic treatment with a combination of high dose glucocorticoids and other immunosuppressive agents most commonly either azathioprine or cyclophosphamide (Morrow et al., 1999).

Cyclophosphamide, mycophenolate, azathioprine and methotrexate may be used to treat major organ manifestations in SLE. However, they inhibit the inflammatory immune response nonspecifically and are therefore associated with a host of side effects that frequently require further therapeutic intervention e.g. corticosteroid induced osteoporosis. Furthermore the management of several manifestations of severe SLE remains controversial. For instance in the treatment of lupus nephritis early studies showed benefit with cyclophosphamide given as intravenous boluses at monthly intervals (for six months then three monthly for two years) added to daily oral prednisolone (Boumpas et al., 1992). This regimen however is associated with infertility (especially in women >30 years) and infection. More recent studies have shown benefit and reduced toxicity with low dose regimens of intravenous cyclophosphamide (500mg monthly for three months) followed by early introduction of azathioprine (Houssiau et al., 2002).

1.3.7 Inadequacies of current treatments in SLE and APS and the need for novel therapies

Despite attempts to improve the side effect profile of cyclophosphamide it is not always effective. A number of novel therapies with improved side effect profiles and/or more specific targeting of the immune system, such as B cell depletion and B cell tolerisation are being developed (reviewed in (Gescuk and Davis, 2002)). Similarly in the treatment of the APS the mainstay of both prophylaxis and active treatment regimes for thrombosis and pregnancy loss is aspirin and anticoagulation. Both of these treatments have potential side effects particularly the risk of haemorrhagic complications with warfarin which is increased when aspirin is taken concomitantly.

It is therefore important to develop new treatments which are both more effective and more accurately targeted to the disease process in APS and SLE. Considerable evidence exists that aPL play a major role in causing the clinical features of APS (reviewed in section 1.2.4). A greater understanding of the structural properties of aPL that determine how they bind to their target antigen may allow the development of new therapies, with hopefully better efficacy and/or better side effect profiles, to block or manipulate interactions between aPL and their major epitopes leading to an amelioration of disease features in the APS.

1.4 ANTIPHOSPHOLIPID ANTIBODIES

1.4.1 Aetiology of aPL

The precise aetiology of aPL remains unclear. A genetic predisposition to aPL production has been described in both family and population studies. The first report suggesting a familial association of the LA described two pairs of siblings with clear evidence of LA (Exner et al., 1980). Subsequent studies have reported that first degree relatives of patients with SLE or PAPS had a higher incidence of aCL (Mackworth-Young et al., 1987; Radway-Bright et al., 2000). Further evidence of a familial form of APS has come from the identification of several kindreds with an increased frequency of aPL and the associated clinical manifestations (Mackie *et al.*, 1987; Matthey *et al.*, 1989; Ford *et al.*, 1990; Dagenais *et al.*, 1992; Bansal *et al.*, 1996).

Various studies have proposed that a genetic predisposition to APS is at least in part to be accounted for by the human leucocyte antigen (HLA) locus. A variety of different HLA class II alleles have been identified. In northern Italian patients with SLE, aCL were found to be significantly associated with HLA DR7 (Savi et al., 1988) whilst in English patients with SLE, HLA DR4 was significantly associated with aCL (McHugh and Maddison, 1989). In neither of these studies was it clear whether any of the patients examined had manifestations of the APS. Two studies of PAPS patients however have also indicated an increased frequency of DR-4 and DR-7 specifically DRw53 (Trabace *et al.*, 1991; Asherson *et al.*, 1992; Camps Garcia *et al.*, 1994) as well as DQw7 (Arnett et al., 1991; Camps Garcia et al., 1994). A recent U.K. study of 83 British Caucasoid APS patients found the DQB1*0604/5/6/7/9-DQA1*0102-DRB1*1302 haplotypes to be significantly correlated with anti-β_2GPI antibodies in the subset of PAPS patients (Caliz et al., 2001). They also found the presence of the DQB1*0303-DQA1*0201-DRB1*0701 haplotype to be associated with APS in all patients (Caliz et al., 2001).

In contrast several large studies have not found an association with DRw53 (Arnett et al., 1999; Galeazzi et al., 2000). Arnett *et al.* (1999) analysed the association of anti-β_2GPI antibodies in a multiethnic group of 262 patients with SLE, PAPS or another ARD. They found that HLA-DR4 haploytpes especially those carrying HLA-DQ8 (DQB1*0302) were associated with anti-β_2GPI antibodies in Mexican Americans and to a lesser extent in whites. In addition they found that the HLA-DRB1*1302;DQB1*0604/0605 haplotype was associated with anti-β_2GPI antibodies primarily in black patients (Arnett et al., 1999). A large European study of 600 SLE patients found that aCL were positively associated with HLA-DRB1*04, -DRB1*07, -DQA1*0201, -DQA1*0301, -DQB1*0302, -DRB3*0301 and that anti-β_2GPI antibodies have a positive association with DQB1*0302 (Galeazzi et al., 2000).

A further layer of complexity is added when other studies are considered which have failed to find an association with HLA antigens at all. Goel *et al.* (1999) studied seven families with 101 family members 30 of whom met their own diagnostic criteria for APS and failed to find linkage in the APS families with HLA or markers for several other candidate genes. Their results were consistent with a genetic basis for disease in APS families which appeared to be inherited in an autosomal dominant pattern (Goel et al., 1999). Close inspection however, of the results of this study reveals that the seven probands were diagnosed with at least one major clinical manifestation (vascular thrombosis and/or recurrent fetal loss) of APS and medium positive titres of aCL or positive LA. In contrast, family members with a medium positive titre aCL or positive LA and minor or no clinical manifestations were diagnosed with APS. Major manifestations of the APS occurred in only six of the 23 "affected" family members. Therefore this study is really an examination of the genetics of aPL rather than the APS.

Many of these apparently conflicting studies are difficult to interpret due to small patient numbers and the use of ethnically diverse patient groups. Further confounding factors from some studies are likely to be the study of aPL positive patients who do not have the APS; the use of different classification criteria to identify APS patients; a lack of patient stratification (primary versus secondary APS) and variation in the measurement of aPL. Overall genetic factors appear to be important in the development of APS and HLA alleles appear to account for only part of this susceptibility. In fact HLA alleles may only determine the susceptibility to produce aPL that are then responsible for the clinical manifestations of APS. The alleles identified thus far may only be apparent because of their linkage disequilibrium with an as yet unidentified primarily involved HLA locus or they could act in cooperation with other genes residing outside the MHC. The search for a more strongly associated polymorphism continues.

A number of different infections and drugs have been implicated as precipitating factors in the generation of aPL. These precipitants however are mostly associated with the formation of cofactor independent IgM aPL. Notable exceptions are findings of $ß_2$GPI dependent antibodies in patients infected with parvovirus B19 (Loizou et al., 1997) and patients taking procainamide (Merrill et al., 1997).

1.4.2 The nature of pathogenic aPL

Not all aPL are associated with pathogenicity. aPL can occur in 1.5-5% of healthy people and may also occur in various medical conditions without causing clinical features of APS (Greaves et al., 2000). The aPL which are found in patients with APS, whether primary or secondary to an ARD differ from those found in healthy people in that they target predominantly negatively charged PL and are directed against a variety of phospholipid binding serum proteins, of which $ß_2$GPI is the most extensively studied and appears to be the most relevant clinically. It has been suggested that the properties of IgG isotype, specificity for negatively charged PL and ability to bind $ß_2$GPI may define a population of pathogenic aPL which are particularly likely to cause the effects on thrombosis that lead to APS (described in section 1.2.5).

1.4.3 The causative role of pathogenic aPL in APS

The most convincing evidence of the pathogenic nature of aPL comes from the induction of typical APS features in naïve mice by both the passive transfer of aPL and active immunization with aPL or $ß_2$GPI (reviewed in (Radway-Bright et al., 1999)). The full range of clinical manifestations seen in human APS however has proved difficult to reproduce in any one single animal model of APS.

There are few models of the thrombosis found in patients with APS. Pierangeli *et al.* (Pierangeli and Harris, 1994; Pierangeli et al., 1996) have shown that when the femoral vein of a mouse is subjected to a pinch stimulus, the size and longevity of the thrombus produced can be enhanced in the presence of aPL which has been passively infused or produced by immunization with $ß_2$GPI/aCL compared to a control IgG antibody.

Intraperitoneal injection of affinity purified IgG aPL (isolated from APS patients) led to endothelial cell (EC) activation by significantly increasing leucocyte adhesion *in-vivo* and the expression of adhesion molecules *in-vitro* (Pierangeli et al., 1999). Monoclonal aPL, induced in mice by immunization with a 15 amino acid (AA) synthetic peptide (called GDKV) that contains the major PL binding region of β_2GPI, have also been shown to enhance thrombus formation and increase leucocyte adherence to EC in a mouse model (Gharavi et al., 1999). A later study of seven maPL derived from two APS patients, found four to be thrombogenic in the pinch stimulus model and three caused more *in-vivo* leucocyte adhesion compared with control IgG mAbs and enhanced adhesion molecule molecule expression in-vitro (Pierangeli *et al.*, 2000). These findings provide direct evidence that aPL are pro-thrombotic but also clearly demonstrate the heterogeneity of these antibodies.

Other models of APS exist, mainly in mice prone to spontaneously develop autoimmune disease. NZWxBXSB F_1 mice produce high titres of aCL and develop degenerative coronary vascular disease with myocardial infarction and thrombocytopenia (Hashimoto et al., 1992). MRL/lpr mice are a lupus-prone strain with a recessive lymphoproliferation gene (lpr) encoding a defective Fas molecule, leading to defective Fas mediated apoptosis. These mice spontaneously develop some features similar to human APS such as high levels of IgG aCL, thrombocytopenia and poor pregnancy outcome (Gharavi et al., 1989) and have histological evidence of thrombosis in the brain (Smith et al., 1990).

More direct evidence of a pathogenic role of aPL in obstetric manifestations of APS comes from the models of passive and actively induced APS. Passive induction entails the intravenous infusion of monoclonal and/or polyclonal aPL into mice to create disease manifestations. Blank *et al.* (1991) infused murine monoclonal aPL and human polyclonal IgG aPL and monoclonal IgM aPL from a patient with PAPS into pregnant ICR mice. They found a lower fecundity rate, increased resorptions index of embryos, lower numbers of embryos per pregnancy and lower weights of embryos and placentae compared to mice infused with murine or human control Ig lacking aCL binding (Blank et al., 1991). Similarly, a significantly higher rate of fetal resorptions with a significant reduction in fetal and placental weight was found in pregnant BALB/c mice passively infused with human monoclonal IgG aPL from a PAPS patient compared to mice infused with a control human monoclonal IgG Ab lacking aPL activity (Ikematsu et al., 1998).

Active induction of APS entails administration of aPL or generation of this Ab in response to an autoantigen, followed by stimulation of the immune system by one of its idiotypes to produce an Ab against it. Consequently Ab directed against the anti-idiotypic Ab may be generated which has the binding characteristics of the original Ab. Following intradermal immunisation of naïve BALB/c mice with human IgM and IgG aPL, the mice have been shown to develop high titres of aPL, prolongation of the activated partial thromboplastin time (aPTT), thrombocytopenia and pregnancy morbidity compared to mice immunised with control Ig lacking aPL activity (Bakimer et al., 1992; Cohen et al., 1993). Bakimer *et al.* (1992) used a human monoclonal IgM aPL generated from a healthy subject following immunisation with diphtheria and tetanus hence the relevance of this study to the APS is less certain. Cohen *et al.* (1993) however used polyclonal aPL purified from a PAPS patient. They found that mice immunised with purified IgG aPL showed low fecundity, increased fetal resorptions with reduced fetal and placental weights compared to controls whilst mice immunised with purified IgM aPL showed only a reduced fecundity and slightly increased fetal resorption rate. Immunisation of naïve BALB/c mice with β_2GPI has also been shown to induce the above features compared to mice immunised with CL (Blank et al., 1994).

1.5 MECHANISMS OF PATHOGENESIS OF THE APS

A satisfactory unifying explanation for the association between aPL and all thrombotic events in APS patients has yet to be defined. The frequent and variable manifestations of thrombosis in APS patients may, in part, be explained by the heterogeneous population of aPL which depending upon their specificity can interfere with different pro-coagulant reactions. Various mechanisms have been proposed and the most consistent and reproducible data suggest that the major mechanisms of hypercoagulability in APS involve up-regulation of the tissue factor (TF) pathway, inhibition of the protein C pathway and the interaction of aPL with the endothelium.

1.5.1 Tissue factor pathway

TF, an inducible cell glycoprotein, is the major initiator of coagulation *in-vivo*. It is expressed in a variety of cells (e.g. EC and monocytes) in response to various stimuli including bacterial lipopolysaccharide (LPS), IL-1 and tumour necrosis factor (TNF). Expression of TF results in formation of a complex between TF and coagulation factor VII on the cell surface, which activates factors IX and X. Activated factor X, in conjunction with activated factor V, then converts prothrombin to thrombin which leads to fibrin formation and platelet activation (Cuadrado et al., 1997).

In-vitro studies have shown that sera from SLE patients with LA induce TF activity in cultured EC (Tannenbaum et al., 1986). A number of other studies have shown that serum, plasma, purified total IgG and anti-β_2GPI antibodies from APS patients enhance TF expression and procoagulant activity on normal monocytes (Kornberg et al., 1994; Reverter et al., 1998). Additionally, using preserved *ex-vivo* monocytes thawed and grown in tissue culture, TF expression was found to be increased in APS patients with a history of thrombosis compared to APS patients without thrombosis; patients with thrombosis and no aPL; and a group of healthy controls (Cuadrado et al., 1997). To clarify the effects of thawing and cell adhesion on monocyte activation the same group have used uncultured fresh mononuclear blood cells and shown that mean levels of TF mRNA were statistically increased in monocytes from PAPS patients with thrombosis compared to six healthy controls (Dobado-Berrios et al., 1999).

The TF/VIIa activity is regulated by tissue factor pathway inhibitor (TFPI) complexed with factor Xa. Up-regulation of the TF pathway could potentially be caused by decreased TFPI activity. Functional anti-TFPI activity has been detected in a subset of PAPS patients (Adams et al., 2001). Furthermore increased factor Xa generation in normal plasma, indicating suppression of TFPI activity, stimulated by aPL isolated from six PAPS patients was lost when patients' IgG was depleted of anti-β_2GPI Ab or when normal plasma was depleted of β_2GPI or TFPI (Salemink et al., 2000). Thus anti-β_2GPI antibodies have been implicated in anti-TFPI activity.

An additional mechanism by which monocyte TF activity may be increased in APS involves the cellular immune response to β_2GPI. It has been shown that CD4+ T cells specific for β_2GPI from APS patients can induce monocyte TF upregulation when cultured in the presence of β_2GPI (Visvanathan et al., 2000).

1.5.2 The protein C pathway

The protein C pathway is an important endogenous antithrombotic mechanism which is initiated when thrombin binds to thrombomodulin on EC (Esmon et al., 1997). Thrombin then loses its procoagulant activity and its ability to activate protein C is markedly enhanced. Activated protein C (APC) acts as an anticoagulant by proteolytically inactivating factors Va and VIIIa with its cofactor protein S, thus limiting the rate of thrombin generation. Various studies have shown that aPL may interfere with the protein C pathway in many different ways with the most convincing evidence indicating inhibition of protein C activation and function by aPL.

A number of publications have observed the inhibition of both protein C activation and function in association with aPL (Marciniak and Romond, 1989; Malia *et al.*, 1990; Borrell *et al.*, 1992). Marciniak and Romond (1989) reported a decreased rate of factor Va degradation in the plasma of 15 patients with LA. Borrell *et al.* (1992) found that IgG fractions from almost all of 21 aPL positive patients with APS, SLE and one asymptomatic donor, showed an inhibitory effect on factor Va degradation by APC when compared with control IgG from normal subjects. Similar results were obtained by Oosting *et al.* (1993) studying purified IgG from 30 patients, of whom 26 had aPL and 23 had thrombosis. They also showed that the antibodies responsible for inhibiting Va degradation were directed against PL bound protein C or protein S (Oosting et al., 1993). Plasma and purified IgG fractions from APS patients may reduce the effect of activated protein C in functional assays for APC resistance leading to an acquired protein C resistance (Male et al., 2001). This anti-APC activity was found to be dependant upon the phospholipid phosphatidylethanolamine (PE) in one study (Smirnov et al., 1995). Even a partial reduction of APC anticoagulant activity is known to be a major thrombotic risk factor (Esmon et al., 1997).

Anti- β_2GPI Ab have been shown to interfere with the protein C pathway although some data are equivocal. β_2GPI itself can inhibit protein C activation by thrombin/thrombomodulin. This was shown using β_2GPI incubated with CL vesicles

(Keeling et al., 1993). Other studies have shown that the binding of protein C to PL is inhibited by purified β_2GPI (Mori et al., 1996) and that aPL recognise protein C only in the presence of β_2GPI (Atsumi et al., 1998). In contrast, another study found β_2GPI to have little or no effect on protein C activation on endothelial cells, in the presence or absence of anti-β_2GPI Ab (Oosting et al., 1991). Whilst the APC resistance of aPL has been shown to be strictly β_2GPI dependent by others (Galli et al., 1998).

There are a number of conflicting reports regarding the presence of aPL directed against thrombomodulin. Several studies using IgG isolated from LA positive plasma found that the Abs were directed against thrombomodulin (Freyssinet *et al.*, 1986; Cariou *et al.*, 1988), whilst other groups have failed to support these observations at all (Watson and Schorer, 1991; Keeling *et al.*, 1993) and one study of IgG from 46 different patients with LA found only two cases with reduced rate of APC formation (Potzsch et al., 1995). Therefore although some aPL may be directed against thrombomodulin their functional effects are unknown and their frequency in the population tested was so low that they cannot be the mechanism that explains the majority of aPL mediated thrombotic events.

There are case reports of acquired protein C or S deficiencies in isolated APS patients β_2GPI (Parke *et al.*, 1992). In general, however, studies in larger populations of patients with APS have failed to show a correlation between decreased protein C levels and the presence of aPL (Hasselaar et al., 1989).

1.5.3 Effects of aPL on vascular EC

Recent work has focused on the aPL-EC interaction. Several different groups have shown aPL to stimulate cultured EC leading to increased expression of adhesion molecules (Del Papa *et al.*, 1995; Simantov *et al.*, 1995; Pierangeli *et al.*, 1999; Pierangeli *et al.*, 2000). In the *in-vitro* studies aPL binding to EC was dependent upon the presence of β_2GPI (Del Papa et al., 1995) and these effects were mediated through the F(ab)`$_2$ fragment of the Ab (Simantov et al., 1995). Using murine *in-vivo* models of microcirculation Pierangeli *et al.* (2000) demonstrated, by direct visualisation, an increased adhesiveness of leucocytes to EC of vessels in the cremaster muscle of mice infused with aPL. They also found the same aPL to enhance thrombus formation *in-vivo* and upregulate the expression of adhesion molecules on cultured human umbilical vein ECs (HUVECs). The importance of certain adhesion molecules – intercellular adhesion molecule 1 (ICAM-1) and P-selectin - was indicated when the same *in-vivo* models of microcirculation and thrombosis were studied using adhesion molecule knockout mice (Pierangeli et al., 2001). In both ICAM-$1^{-/-}$ mice and ICAM-$1^{-/-}$/P-selectin$^{-/-}$ mice, IgG aPL affinity purified from an APS patient did not increase leucocyte adhesion to ECs nor did these Ab enhance thrombus formation (Pierangeli et al., 2001). Thus aPL appear to induce a proadhesive and proinflammatory phenotype. Interestingly fluvastatin, a fully synthetic hydroxymethylglutaryl coenzyme A reductase inhibitor, has been shown to inhibit EC adhesion molecule expression on cultured HUVECs induced by human aPL (Meroni et al., 2001). More recently, fluvastatin has been shown to significantly diminish aPL mediated thrombosis and EC activation in the *in-vivo* models of microcirculation and thrombosis described above (Ferrara et al., 2003).

E-selectin up regulation in ECs activated by aPL binding to β_2GPI has been shown to be dependant on translocation of a transcription factor, nuclear factor κB (NF-κB) in a manner comparable to that induced by proinflammatory cytokines (IL-1β and TNFα) or by bacterial LPS (Meroni et al., 2001). It is known that LPS and IL-1 interact with membrane proteins, namely toll-like receptors (TLRs) and IL-1 receptors, which share a homologous cytoplasmic signalling domain, the toll/IL-1 receptor (TIR) domain and use the same intracellular mediators in their activation pathway. The adapter molecule myeloid differentiation protein (MyD88) is the first protein that associates with both the TLRs and IL-1R through its TIR domain. Recently, Raschi *et al.* (2003) have proposed that aPL activate EC through a TLR and the MyD88 pathway after experiments in which they co-transfected immortalised human microvascular EC with dominant negative constructs of different components of the TLR signalling pathway. They also found EC activation by human anti-β_2GPI Ab produced a signalling cascade comparable to that induced by bacterial LPS which is known to act via TLR4 (Raschi et al., 2003).

1.5.4 aPL and the development of atherosclerosis

An emerging concept is that aPL have an effect on the development of AS. Low density lipoprotein receptor deficient (LDL-RD) mice fed a high cholesterol diet develop AS and apo-E deficient mice develop spontaneous AS. Immunisation of either of these mouse strains with $ß_2$GPI leads to the production of high titers of anti-$ß_2$GPI antibodies, whilst controls of non-immunised mice or mice immunised against ovalbumin do not develop these antibodies (George et al., 1998a; George et al., 1999). As well as a raised titer of anti-β_2GPI antibodies, larger AS lesions were found in the immunised animals which contained abundant CD4 cells also (George et al., 1998a; George et al., 1999). Similarly when LDL-RD mice were immunised with aCL they developed high titers of anti-anti-aCL, that had the same binding characteristics as the original antibody used for manipulation, and had increased AS compared with control mice immunised with normal human IgG (George et al., 1997). Further evidence of the association of aPL and AS in mice comes from experiments in which lymphocytes transferred from β_2GPI immunised LDL-RD mice by intraperitoneal injection into syngenic LDL-RD mice led to enhanced AS in the recipients (George et al., 2000).

In the presence of aPL, β_2GPI increases the uptake of oxidised LDL by macrophages, which is an important step in the formation of AS (for review see (Petri, 1998)). It has been suggested that β_2GPI dependent aPL may cause AS by direct activation of the vascular endothelium (Simantov *et al.*, 1995; Del Papa *et al.*, 1997; Pierangeli *et al.*, 1999), increased clearance of oxidised LDL (Hasunuma et al., 1997), or by reduced paraoxonase activity (Delgado Alves et al., 2002). Paraoxonase is a circulating anti-oxidant, levels of which are reduced in APS. This reduction may lead to increased oxidation of LDL.

Despite this experimental evidence, the findings from clinical studies of a link between the presence of carotid AS and aPL are conflicting. One study found a statistically significant association (Ames et al., 2002) between IgG aCL titre and the intima media thickness (IMT) of carotid arteries and another found a trend towards a higher prevalence of carotid plaque in aPL positive patients (Roman et al., 2001). Several groups however, have not found any association between aPL and carotid or coronary AS (Asanuma et al., 2003; Roman et al., 2003; Vlachoyiannopoulos et al., 2003). Notably of those studies which did support an association between aPL and AS, Ames *et al.* (2002) did not use a control group and the other was a pilot study (Roman et al., 2001) which subsequently did not support the trend towards a positive association in a larger group of patients (Roman et al., 2003). Thus a clinical association between the presence of carotid AS and aPL has not yet been conclusively proven and requires further clinical studies.

1.5.5 Other pathogenic mechanisms of aPL

Other hypotheses for pathogenic mechanisms of aPL have been postulated. aPL have been shown to interfere with the production and release of prostacyclin by endothelial cells, disrupt the Annexin V anticoagulant shield, both inhibit and induce platelet activation, impair fibrinolytic mechanisms and interfere with antithrombin III activity (for reviews see (Santoro, 1994) and (Rand, 2002)).

1.5.6 The role of T cells and cytokines in the APS

Given the importance of T cells and cytokines in the humoral immune response, explained in section 1.1.6, it is not surprising that evidence is emerging which implicates these molecules in the pathogenesis of the APS.

Cytokine abnormalities have been implicated in the pathogenesis of fetal loss, one of the major manifestations of the APS. In normal pregnancies IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) have been shown to promote placental development and fetal growth (Wegmann, 1989) and the concentration of IL-3 rises, to peak at delivery (Fishman *et al.*, 1992b). Low levels of IL-3 have been found both in APS patients (Shoenfeld *et al.*, 1998b) and in BALB/c mice with high titers of aPL and pregnancy morbidity following active immunisation with a human maPL (Fishman *et al.*, 1992a). Furthermore administration of murine recombinant IL-3 to pregnant ICR mice which had been passively injected with aPL led to a dramatic reduction in the fetal resorptions rate and reduction in severity of thrombocytopenia (Fishman et al., 1993). It has also been suggested that the beneficial effect of low dose aspirin in preventing the clinical manifestations of APS may in part be mediated by its ability to stimulate IL-3 production (Fishman et al., 1996). Similarly, Ciprofloxacin (a broad spectrum quinolone antibiotic) has been shown to decrease pregnancy loss in association with an increase in IL-3 and GM-CSF production (Riesbeck et al., 1994). However the exact role of IL-3 in the pathogenesis of the APS remains unclear.

Since autoantibody production by B cells generally requires T cell help, it is not surprising that T cells appear to have a prominent part in the pathogenesis of APS. High titers of aPL and pregnancy morbidity have been shown to be induced in total body irradiated naïve BALB/c recipient mice following infusion of T cells originating from naïve mice that were actively immunised with a murine maPL (Blank et al., 1995). Similar features of the APS were not seen when T cells were taken from donor mice immunised with a control IgG aPL which lacks CL binding (Blank et al., 1995). Anti-CD4 mAbs, given before or after induction of experimental SLE and APS in BALB/c mice, can prevent the development of autoantibody production, including aPL as well as the clinical manifestations of the disease (Tomer et al., 1994).

Circulating $ß_2$GPI specific CD4+ T cells have been demonstrated in 8 out of 18 patients with APS studied but not found in healthy individuals or patients with other ARD or aPL positive patients who did not have the APS (Visvanathan and McNeil, 1999). T cells that are autoreactive to $ß_2$GPI have been found in APS patients and healthy individuals (Hattori et al., 2000). Native $ß_2$GPI however, did not induce a proliferative response in peripheral blood T cells which were only stimulated by a reduced form of $ß_2$GPI. This discrepancy was thought to have occurred because T cell tolerance to peptides generated through normal processing of $ß_2$GPI is induced because of the abundance of $ß_2$GPI in the circulation. Thus it was considered likely that the reduced form of $ß_2$GPI expresses cryptic peptides that are able to stimulate T cells. How much, if any of this reduced form of $ß_2$GPI is found in-vivo is unknown, thus the relevance of these findings to patients with APS is uncertain. Furthermore the authors admit, that the mechanism by which exposure to cryptic peptides may result in the activation of $ß_2$GPI reactive T cells in APS patients is unknown (Hattori et al., 2000).

To examine antigen recognition profiles and Th cell activity in $ß_2$GPI reactive T cells further, the same group generated 14 CD4+ T cell clones specific to $ß_2$GPI from three patients with APS (Arai et al., 2001). At least four distinct T cell epitopes were identified, but the majority of the $ß_2$GPI specific T cell clones responded to a peptide encompassing AA residues 276 to 290 of $ß_2$GPI containing the major PL binding site. Ten of 12 $ß_2$GPI specific T cell clones stimulated peripheral blood B cells that produced anti-$ß_2$GPI Ab in the presence of recombinant $ß_2$GPI. Th cell activity was found exclusively in T cell clones capable of producing IL-6.*In-vitro* anti-$ß_2$GPI Ab production induced by T cell clones was inhibited by either anti-IL-6 or by the anti-CD40 ligand monoclonal antibody. In addition, exogenous IL-6 augmented anti-$ß_2$GPI Ab production in cultures of B cells with T cell clones lacking IL-6 expression. The results of this study indicate that $ß_2$GPI specific CD4+

T cells in APS patients preferentially recognise the antigenic peptide containing the major PL binding site and have the capability to stimulate B cells to produce **anti-β_2GPI** Ab via expression of IL-6 (Arai et al., 2001).

In contrast, another group generated CD4+ T cell lines reactive with β_2GPI peptides from three of 18 ARD patients (five with APS) who were all carrying anti-β_2GPI Ab. They found the major T cell epitope to reside in a fragment containing codons 244-264 and a leucine at position 247. This epitope however was also found in a CD4+ T cell line derived from one of two healthy control subjects who did not have anti-β_2GPI Ab (Ito et al., 2000).

Evidence of T cells in the lesions of AS (George *et al.*, 1999; George *et al.*, 2000) and increased monocyte TF activity found in the APS (Visvanathan et al., 2000) has previously been described in sections 1.5.4 and 1.5.1 respectively.

1.6 THE IMPORTANCE OF β_2GPI IN APS

Although β_2GPI is a major target antigen in the pathogenesis of the APS (described in section 1.2.5), the exact nature of the aPL-β_2GPI interaction remains a matter of some debate. Opinion is divided as to whether pathogenic aPL are directed against the β_2GPI-PL complex (Shoenfeld *et al.*, 1998a), a cryptic epitope revealed on β_2GPI by binding to PL (or certain synthetic surfaces) (Koike et al., 1998), or whether they bind directly to an increased density of β_2GPI immobilised upon PL or gamma irradiated plates (Roubey et al., 1995), see Figure 1.2. The location of the major epitopes on β_2GPI is also unclear and evidence has been published supporting the existence of epitopes in various different regions of β_2GPI.

1.6.1 β_2GPI – Molecular structure and function

β_2GPI is a 50kDa (Schultze et al., 1961) single chain polypeptide, 326 amino acid residues long with five oligosaccharide attachment points (Lozier et al., 1984; Steinkasserer et al., 1991). The protein is composed of five common structurally related repeats known as domains which are classed as members of the short consensus repeat or complement control protein (CCP) super family (Reid and Day, 1989). Each domain is approximately 60 amino acid residues in length with two intra-domain disulphide bonds. The first four domains are typical CCP domains but domain V is aberrant, containing 82 amino acids and

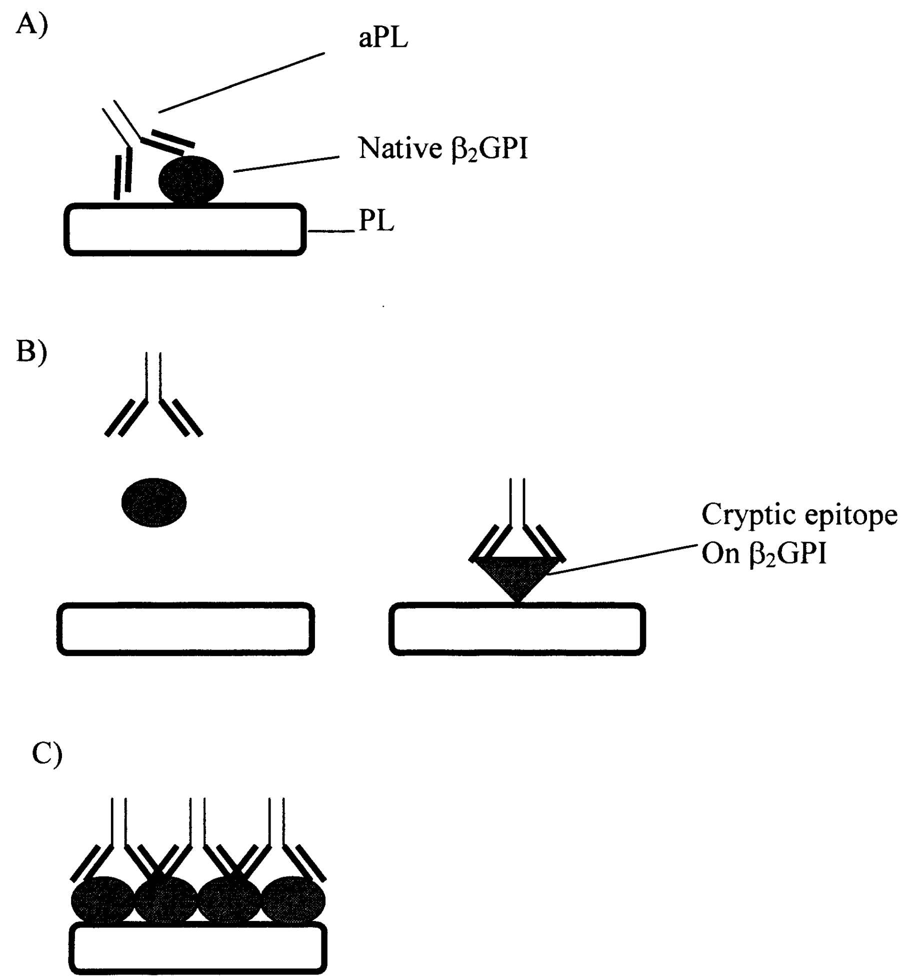


Figure 1.2 Schematic representations of various hypotheses regarding aPL-β_2GPI interaction

Pathogenic aPL are depicted binding to: - A) the β_2GPI-PL complex; B) a cryptic epitope revealed on β_2GPI by binding to PL (or certain synthetic surfaces); C) an increased density of β_2GPI immobilised upon PL (or certain synthetic surfaces).

a long carboxyl terminal tail which is cross linked by an additional disulphide bond. The crystal structure of ß$_2$GPI has been described by two independent groups (Bouma et al., 1999; Schwarzenbacher et al., 1999).

The precise physiological function of human ß$_2$GPI remains unclear. *In-vitro*, ß$_2$GPI binds to negatively charged phospholipids (such as CL and phosphatidylserine (PS)), inhibits contact activation of the intrinsic coagulation pathway (Schousboe, 1985), platelet prothombinase activity (Nimpf et al., 1986), adenosine diphosphate (ADP) mediated platelet aggregation (Nimpf et al., 1987) and aids clearance of oxidised LDL, (Hasunuma et al., 1997). *In-vivo*, ß$_2$GPI is involved in the clearance of apoptotic bodies (Pittoni et al., 2000) and liposomes (Chonn et al., 1995) as well as interfering with the protein C pathway (Roubey, 1996). ß$_2$GPI adheres to resting human EC. Subsequent binding of circulating aPL led to EC activation (Del Papa et al., 1997) (for review see (Meroni et al., 1998)). Thus ß$_2$GPI appears to promote clearance of certain products of oxidation and prevent thrombus formation (for review see (Inanc et al., 1997)). Binding of aPL to epitopes on ß$_2$GPI may interfere with these functions, and therefore promote thrombosis. The possible role of ß$_2$GPI on the development of AS has been discussed in section 1.5.6.

1.6.2 Evidence in favour of a cryptic epitope upon ß$_2$GPI

Several groups have found monoclonal (Matsuura et al., 1994) and polyclonal aPL (Chamley et al., 1999) bind directly to ß$_2$GPI coated on irradiated, but not plain, polystyrene ELISA plates in the absence of CL, even when the concentration of ß$_2$GPI on the plain plates was higher. Thus these authors felt that a conformational change in ß$_2$GPI was required to explain the increased binding of aPL to irradiated plates.

Indirect methods using infrared spectroscopy (Borchman et al., 1995), spectrophotometry to produce a circular dichroism spectrum (Subang et al., 2000) and microcalorimetry (Hammel et al., 2001) have all been used to infer an alteration in the structure of ß$_2$GPI upon binding to PL. Subang *et al.* (2000) demonstrated a conformational change in ß$_2$GPI upon binding to CL but not other anionic PL such as phosphatidylglycerol (PG) and PS. Despite this, ß$_2$GPI complexes with PG and PS were still found to be immunogenic, leading to the production of aPL and LA in BALB/c mice.

Matsuura *et al.* (2000) showed no binding of polyclonal or monoclonal human aPL or monoclonal murine aPL to plasmin cleaved β_2GPI on polyoxygenated plates. Binding of β_2GPI to the plates was unaffected by plasmin cleavage, as measured by binding to murine anti-human β_2GPI. Since this experiment measured binding of aPL to β_2GPI in the absence of PL, these results were taken as evidence that proteolytic cleavage of β_2GPI between Lys-317 and Thr-318 does not simply reduce binding to PL but also prevents exposure of cryptic epitopes for aPL (Matsuura et al., 2000).

1.6.3 Evidence against a cryptic epitope

Roubey *et al.* (1995) compared the binding of aPL to β_2GPI bound to plain and irradiated plates and found that aPL binding was detected only when the amount of β_2GPI coated on the high binding plates exceeded that which could be coated to the plain plates. Inhibition experiments demonstrated little detectable binding of aPL to physiologic concentrations of fluid phase β_2GPI or a low density of immobilised β_2GPI. Binding of monovalent Fab` fragments of aPL to $\beta_2$GPI on irradiated plates was substantially less than that of bivalent F(ab`)$_2$ fragments (Roubey et al., 1995). Thus they argued that aPL which bind to β_2GPI are of low affinity, requiring bivalent binding which only occurs when β_2GPI is "clustered" upon an irradiated plate or anionic PL surface and does not require the exposure of a cryptic epitope on β_2GPI .

The striking differences between the results obtained by Roubey *et al.* (1995) and Chamley *et al.* (1999) may be explained in a number of ways. Firstly, the two groups used different techniques to quantify the amount of β_2GPI bound to the plates and carried out their experiments at significantly different coating concentrations. Secondly, different antibodies were tested in the two experiments. It is possible that some aPL require the presence of a cryptic epitope for binding to β_2GPI, whereas others do not.

An insect expression system was used to produce a variant of β_2GPI, which dimerises spontaneously (Sheng et al., 1998). This variant bound polyclonal human aPL better than wild-type β_2GPI on non-irradiated plates, irradiated plates and in the fluid phase. Bivalent F(ab`)$_2$, but not monovalent Fab`, fragments could bind the dimerising variant (Sheng et al., 1998). These experiments again suggested the importance of bivalent binding of low affinity aPL to clustered ß$_2$GPI. It is possible, however, that the process of dimerisation itself might lead to exposure of a cryptic epitope.

Using surface plasmon resonance (SPR) the interactions of five monoclonal murine aPL with ß$_2$GPI have been studied (Regnault et al., 1999). Molar ratios of the monoclonal aPL binding to ß$_2$GPI immobilised upon sensor chips were approximately 0.5, indicating bivalent binding.

1.6.4 Conclusion - is the epitope cryptic?

The binding of aPL to ß$_2$GPI upon certain synthetic surfaces in the absence of PL can be interpreted both for and against a cryptic epitope for aPL upon ß$_2$GPI. Increased binding of aPL to mutant forms of "dimerised" ß$_2$GPI, plus a lack of binding of monovalent aPL Fab` fragments, confirm that many aPL are intrinsically low affinity antibodies for which bivalent binding is required upon clustered ß$_2$GPI.

It is important to recognise that the ideas of cryptic epitopes and high-density binding are not mutually exclusive. A particular aPL may bind preferentially to a cryptic epitope exposed when ß$_2$GPI binds to an irradiated plate but may also be capable of binding to native ß$_2$GPI when presented at sufficiently high density. Overall, however, the evidence in favour of the importance of binding to non-cryptic epitopes currently seems to be the most persuasive, because it has been obtained using a wider range of techniques and antibodies.

1.6.5 Which domains of ß$_2$GPI contain major epitopes for aPL?

During the last eight years evidence has been published demonstrating specificity of aPL for epitopes on all five domains of ß$_2$GPI which is summarised in Table 1.5 and discussed in light of the recently discovered three-dimensional crystal structure of ß$_2$GPI. Lack of patient stratification and control groups plus limited statistical power is commonplace amongst these studies, making it difficult to decide whether the conclusions are likely to be applicable to the majority of patients with APS.

1.6.5.1 Domain V

It is now generally accepted that domain V contains the major site of binding to PL, but it is much less clear whether that domain interacts with aPL .

Author (reference)	Method	Origin of aPL	Original conclusion	Reassessment
Hunt *et al.* (1993)	Plasmin cleaved β_2GPI	PAPS‡ sera	D V – PL binding & cofactor activity	Crystal structure reveals D V to be
Hunt & Krillis (1994)	D V peptides	SLE & PAPS* sera	D V – PL binding (CKNKEKKC) & aPL binding (intact Lys 317-Thr 318)	intimately involved in PL binding
Wang *et al.* (1995)	D V peptides	PAPS* maPL	D V – PL binding (CKNKEKKC) & aPL binding (? exact site)	D V deleted mutants do bind aPL
Sheng *et al.* (1996)	Mutagenesis Lys - 284, 286, 287	APS*§ sera	Lys - 284, 286, 287 critical for PL binding	
Igarashi *et al.* (1996)	DM	PAPS* maPL & Murine maPL	D V – PL binding D I-IV, favoured D IV aPL binding	Crystal structure reveals domain III and IV to be heavily glycosylated.
George *et al.* (1998c)	DM	PAPS & SAPS*† sera, murine maPL	D V – PL binding D I-IV aPL binding	D IV produced from insect cells may be
Blank *et al.* (1999a)	Hexapeptide phage display	PAPS* & normal sera	D I-II interlinker, D III, D IV contain epitopes for aPL binding	less glycosylated than human β_2GPI
Iverson *et al.* (1998)	DM – glyhis6 tag	PAPS & SAPS*† sera	D I binds aPL	Crystal structure reveals D I and II to be
McNeeley *et al.* (2001)	SPR, DM	APS*§ sera	D I binding in 80% sera	the most accessible domains for aPL binding.
Guerin *et al.* (2000)	Elastase & Protease cleavage β_2GPI	APS‡§ sera	D V cleavage lost aPL binding 91% sera D I cleavage 50% reduction in aPL binding 81% sera	Most persuasive evidence for D I
Sheng *et al.* (2001)	LA activity domain specific aPL	murine maPL	D I binding aPL prolong dRVVT & KCT > than - D II binding aPL prolong KCT	Pathogenic aPL contain clusters of positively charged residues. In D I there is a surface exposed pocket of negative
Reddel *et al.* (2000)	mutagenesis D I β_2GPI	APS*§ & aPL +ve sera, murine maPL	D I binds aPL	charge

Table 1.5 Summary of experimental data linking various domains of β_2GPI with aPL binding

Abbreviations:- maPL – monoclonal aPL; D – Domain; KCT – kaolin cephalin clotting time; SPR – surface plasmon resonance; for others see text.

Symbols:- * - APS classification criteria not applied; † - APS secondary to SLE; ‡ - APS classification criteria applied; § - no distinction primary or secondary APS.

The lysine-rich sequence Cys 281–Lys-Asn-Lys-Glu-Lys-Lys-Cys-288 (CKNKEKKC) is particularly crucial in binding to PL. β_2GPI loses its ability to bind PL, or to act as a cofactor for aPL/PL binding, when the number of lysines in this region is reduced by mutagenesis (Sheng et al., 1996). Peptides containing the CKNKEKKC sequence inhibit binding of purified aPL in a CL-ELISA. The importance of domain V in binding to PL is underlined by the finding that a preparation of β_2GPI cleaved between residues Lys-317 and Thr-318 in domain V completely lost its ability to bind negatively charged PL and to act as a cofactor for affinity purified aPL from "autoimmune patients" (Hunt et al., 1993). It was suggested that this cleavage also reduced the binding of β_2GPI to polyclonal human aPL (Hunt and Krilis, 1994), but mutations in the CKNKEKKC sequence did not affect this binding (Sheng et al., 1996). These data gave conflicting messages as to whether domain V is important in the interaction between β_2GPI and aPL.

Gharavi and colleagues demonstrated that immunisation of mice with purified whole human or bovine β_2GPI induced aPL production (Gharavi et al., 1992).Subsequent work by the same group has implicated the domain V CKNKEKKC sequence in this process. aPL were induced in mice by immunisation with a synthetic peptide (called GDKV) spanning Gly274-Cys288 of domain V in β_2GPI. Mice immunised with the peptide and carrier protein complexes produced significantly higher levels of aPL than those mice immunised with either the peptide or carrier protein alone. The GDKV induced maPL were shown to enhance thrombus formation and increase leucocyte adherence to EC in a mouse model (Gharavi et al., 1999). Furthermore, this group have recently shown that pathogenic aPL can be generated by immunising mice with a peptide, derived from human cytomegalovirus, that resembles the GDKV peptide (Gharavi et al., 2002).

However, consideration of the crystal structure of β_2GPI reveals that the sequence Ser-311-Lys-317 forms a hydrophobic loop acting as a membrane stabiliser for the positively charged CKNKEKKC PL binding region. This region is so closely apposed to the PL membrane that it is unlikely to be a major epitope for binding to aPL under physiological conditions. Although previous studies suggested that regions of domain V outside CKNKEKKC might be responsible for binding of aPL (Hunt and Krilis, 1994; Wang et al., 1995), this now seems unlikely. Those regions would be sterically hindered from interaction with aPL, because they are not surface-exposed, or because they are in close proximity to the PL membrane bound to domain V. In light of the crystal structure it would seem more likely for the aPL binding site to be contained in another domain.

1.6.5.2 Domain IV

Two groups have studied mutant forms of ß$_2$GPI in which various domains were deleted and expressed in insect cells (Igarashi *et al.*, 1996; George *et al.*, 1998c). Both groups confirmed that domain (D) V was essential for binding to CL whilst binding to human or murine monoclonal aPL took place in the absence of DV. The mutant DI-IV bound the murine antibody WB-CAL-1 better than either whole ß$_2$GPI or mutant DI-III. Unusually, WB CAL-1 was able to bind to DI-IV upon plain polystyrene plates so this mutant was thought to have undergone a conformational change following the deletion of domain V thus exposing an epitope for aPL. It was concluded that domain IV contains a major epitope for binding of these aPL. Interestingly the authors did not demonstrate binding of WB CAL-1 to any domain deleted mutant (DM) lacking DI and conversely DM peptides containing only DI or DI-II were not made or tested. They concluded that autoimmune aPL recognise an epitope in domain IV of ß$_2$GPI (George *et al.*, 1998c). This conclusion would have been more compelling if DII-V, DIII-V and DIV-V had been shown to support aPL binding.

Pursuing this hypothesis, another group produced four mutant forms of ß$_2$GPI in which point mutations were made in domain IV (Koike et al., 2000). The binding of human maPL and polyclonal sera from 30 anti-ß$_2$GPI antibody positive APS serum samples to these mutant forms of ß$_2$GPI immobilised upon microtitre plates was greatly reduced in comparison to wild-type ß$_2$GPI. However, it has subsequently been shown (Iverson et al., 2002) that these mutant forms of ß$_2$GPI do bind aPL in the fluid phase and that the results are critically dependent upon the type of ELISA plate used. Therefore the mutations in domain IV alter the interaction of ß$_2$GPI with particular ELISA plates rather than affecting an epitope for binding of aPL. Thus, like domain V, experimental evidence currently does not favour the idea that domain IV contains the major epitopes for the interaction between ß$_2$GPI and aPL

1.6.5.3 Domain III and Domain I-II linker region

Using a phage display library three peptides were identified that react specifically with the three human IgM β_2GPI dependent maPL ILA-1, ILA-3 and H3 (Blank et al., 1999a). These aPL were isolated from a patient with APS and had previously been shown to cause EC activation and induce increased fetal resorption, thrombocytopenia and prolonged aPTT in pregnant BALB/c mice (George *et al.*, 1998b). The peptides bore sequence homology to regions of β_2GPI in the interlinker of domain I-II, domain IV and domain III respectively. All three peptides specifically inhibited *in-vitro* and *in-vivo* biological functions of the corresponding maPL. Exposure of EC to ILA-1, ILA-3 and H3 in the presence of their corresponding peptides inhibited *in-vitro* EC activation. *In-vivo*, the ability of each antibody to induce features of APS in pregnant BALB/c mice was reduced if the corresponding specific peptide was administered post mAb (Blank et al., 1999a).

When affinity purified polyclonal β_2GPI dependent aPL isolated from patients with APS were examined, different fractions of antibodies were identified that specifically recognise each peptide. Sera from only 10 of 43 patients, however, were found to contain Abs to one, two or all three of the peptides. The amounts of the peptide specific anti-β_2GPI fractions varied between 0.4 and 43% of the total anti-β_2GPI Abs identified in each of these 10 patients. The majority of the human anti-β_2GPI must therefore bind to epitopes other than these peptides (Blank et al., 1999a).

The crystal structure shows that domains III and IV are both heavily glycosylated in native β_2GPI. Insect cells however, do not glycosylate proteins the same way as mammalian cells. Recombinant whole β_2GPI expressed in insect cells has an estimated molecular mass of 43kD as opposed to 50kD for native β_2GPI purified from human serum, presumably due to differences in the oligosaccharide chains attached by glycosylation (Igarashi *et al.*, 1996; George *et al.*, 1998c). Hence domain IV in β_2GPI produced from insect cells may be more accessible for binding to aPL than domain IV in human β_2GPI in vivo. This is an important caveat in interpreting data on domains III and IV derived from experiments using the insect expression system.

1.6.5.4 Domain I

Iverson *et al.* (1998) studied binding of DMs to affinity purified polyclonal human aPL from 11 patients (9 of whom had APS). All 11 aPL bound strongly to DI-IV with significant but more variable binding to other DMs containing domain I. In contrast the DMs in which domain I had been deleted showed little or no specific binding to aPL. In the fluid phase, binding of aPL from all 11 patients to ß2GPI plates was inhibited by DMs containing domain I, but not by DMs which lacked domain I (Iverson et al., 1998). These results support the presence of an epitope for aPL residing in a surface exposed region of domain I, which does not need to be revealed by a conformational change.

Iverson *et al.* (1998), Igarashi *et al.* (1996) and George *et al.* (1998b) all studied similar DMs produced from the same insect expression system. Each showed that the DI-IV mutant bound aPL better than any other DM containing DIV. Binding to the DI-III mutant was shown by Iverson *et al.* (1998) and Igarashi *et al.* (1996), but not by George *et al.* (1998b). If domain IV does contain the major epitope for aPL then the mutants DII-V, DIII-V, and DIV-V would be expected to bind aPL to variable degrees. These mutants were either not examined (Igarashi *et al.*, 1996; George *et al.*, 1998c), or found not to bind aPL (Iverson et al., 1998). Thus the data of Iverson *et al.* (1998) complements and extends that of the other two groups and clearly implicates domain I in the binding of aPL particularly as they found that the DI-II and DI mutants both bound aPL.

DM may be subject to misfolding; hence failure of an aPL to bind to a DM may occur because an epitope has been hidden rather than deleted. Single point mutations of specific nucleotides in the DNA encoding $ß_2$GPI may be less likely to alter protein structure. Reddel *et al.* (2000) have studied DMs and whole $ß_2$GPI with single point mutations in domain I. They demonstrated that monoclonal murine $ß_2$GPI-dependent aPL and polyclonal aPL from 18 out of 21 human sera did not bind to DMs lacking domain I. In addition charge altering mutations in domain I of Gly to Glu at position 40 and Arg to Gly at position 43 both lead to a decreased binding to the majority of sera tested. The charge conserving mutation Thr to Ala at position 50 did not show a consistent pattern of altered binding to these sera (Reddel et al., 2000).

Subsequently the Arg to Gly mutation at position 43 has been shown to lead to a reduction in binding to maPL derived from patients with APS (Iverson et al., 2002). These results were reproduced in the solid and fluid phase regardless of the type of ELISA plate used, implying that this mutation is really altering an epitope bound by these maPL. In contrast reduced binding with DIV point mutants was only seen when they were coated on certain types of plate.

Serum samples from a large cohort of 106 patients with APS have been tested for binding to ß$_2$GPI by SPR. These samples revealed a significantly greater interaction with immobilised native ß$_2$GPI in comparison with a DII-V mutant, lacking domain I. Eighty percent of the patient samples exhibited a marked preference for whole ß$_2$GPI containing domain I with almost half of the total patient samples showing an almost complete lack of binding to the DII-V mutant (McNeeley et al., 2001).

Overall, evidence that domain I contains the major epitopes is more robust and more convincing than that for the other domains. This evidence is supported by consideration of the crystal structure.

1.6.6 Consideration of the crystal structure of ß$_2$GPI

The crystal structure (Bouma et al., 1999; Schwarzenbacher et al., 1999) shows a J shaped molecule, with domain V interacting with PL. The solution structure of ß$_2$GPI (Hammel et al., 2002) however, reveals an S-shaped model of human ß$_2$GPI, in which additional carbohydrate residues missing from the crystal structure were modelled and the angle between domain I and domain II was rotated. The corrections evident in the solution structure do not alter the conclusions described below.

Domain V is seen to be intimately involved in binding anionic PL or certain synthetic surfaces and domain IV and III are both heavily glycosylated so even surface exposed residues are shielded by the glycan moieties. Thus the most easily accessible domains for aPL binding would appear to be domain I and II. A detailed examination of surface exposed residues in domain I reveals a region that is rich in negatively charged residues. This region contains two Asp residues at positions 8 and 9 and three Glu residues at positions 23, 26 and 27, which are in close approximation to each other upon the surface of the molecule with only one salt bridge being formed from Glu 26 to a Lys at position 19. Such a pocket of negative charge may be of significance because sequence analysis of pathogenic aPL has shown a clustering of positively charged arginine and lysine residues in their antigen binding regions (Giles et al., 2003b).

1.7 ORGANISATION AND EXPRESSION OF IMMUNOGLOBULIN GENES

Consideration of how aPL may cause the APS, therefore shows that there are a number of possible mechanisms, described in section 1.5. Not all aPL however, are equally capable of causing pathogenesis by these mechanisms. Evidence from clinical and laboratory studies identifies those antibodies which bind negatively charged PL and β_2GPI as being particularly important in pathogenesis of APS. Within β_2GPI domain I is a site of particularly important epitopes of which the surface exposed pocket of negative charge, described in section 1.6.6, may be one.

The work described in this thesis aims to investigate the structural characteristics of aPL which confer these important binding properties. To do this, it is first necessary to consider the basic structure of the genes encoding the antibody binding site

1.7.1 Immunoglobulin gene rearrangement in B cells

In the mammalian immune system the total number of antibody specificities (known as the repertoire) available to an individual is approximately 10^8 to 10^{11} (Berek and Milstein, 1988). Before sequence analysis of Ig genes was possible there were two main theories as to the origin of Ig diversity. The germline theory held that there is a separate gene for each different Ig chain and that the antibody repertoire is largely inherited. In contrast somatic diversification theories proposed that the observed repertoire is generated from a limited number of inherited V region sequences that undergo alteration within a B cell during the individual's lifetime. In 1965 elements of both theories were combined (Dreyer and Bennett, 1965) and it was proposed that each chain of the Ig molecule would be encoded by combining one of a small group of C region genes with a V region gene chosen from a much larger and more diverse population.

Sequence analysis of cloned mouse V_λ (Tonegawa et al., 1977; Tonegawa et al., 1978), V_κ (Sakano et al., 1979; Schilling et al., 1980) and V_H (Schilling et al., 1980) showed that the variable region of the H chain is encoded by three segments in the order V-D-J (variable-diversity-joining) whilst the L chain is encoded by two fused gene segments in the order V-J (variable-joining).

In L chains the V gene segment encodes most of the V domain apart from most of FR4 (up to 13 amino acids) which is encoded by the J gene segment. Similarly in H chains most of the V domain is encoded by the V gene segment apart from most of CDR3 which is encoded by the D and the J segments and therefore has the potential to be more variable than the other regions. The J segment of the H chain also encodes FR4. The entire C region of each chain is encoded by the C gene (Tonegawa, 1983).

These Ig gene segments undergo a process of rearrangement in pre-B cells (early B cell precursors that do not express functional membrane bound Ig) to produce complete Ig H and L chains as described above. DNA rearrangements are guided by conserved noncoding DNA sequences which consist of a conserved palindromic heptamer (5`CACTGTG3`) which is always contiguous with the coding sequence and a conserved nonamer (5`GGTTTTTGT3`). Separating the heptamer and nonamer is a nonconserved region known as the spacer which is either 12 or 23 nucleotides long. One heptamer-spacer-nonamer unit is located 3` to each V gene segment, 5` to each J gene segment and on both sides of the D gene segment (Tonegawa, 1983). The spacer varies in sequence but its conserved length corresponds to one or two turns of the DNA double helix (Early et al., 1980). The heptamer-nonamer sequences are called recombination signal sequences (RSS) since they directly flank each germline V, D and J segment and seem to signal exactly where the germline DNA should be broken during rearrangement.

All V_κ, J_λ (Sakano et al., 1979; Tonegawa, 1983) and D (Kurosawa and Tonegawa, 1982) spacers are 12bp long whilst almost all V_λ, J_κ, V_H and J_H spacers are 23bp long (Sakano et al., 1979; Tonegawa, 1983). Recombination only occurs between gene segments located on the same chromosome and generally follows the rule that only a gene segment flanked by a RSS with a 12bp spacer can be joined to one flanked by a 23bp spacer. This phenomenon is known as the 12/23 rule and explains why some arrangements are allowed (e.g. V-D, D-J, $V_\lambda J_\lambda$) whilst others are not (e.g. V-V or $V_H J_H$) (Early et al., 1980). This process of rearrangement was first described in mice and subsequently confirmed in humans when the first sequences of V_H (Matthyssens and Rabbitts, 1980) D_H (Siebenlist et al., 1981), J_H (Ravetch et al., 1981), V_κ (Bentley and Rabbits, 1980), V_λ (Brockly et al., 1989), J_λ (Vasicek and Leder, 1990) and J_κ (Hieter et al., 1980) were cloned.

1.7.2 The mechanism of immunoglobulin variable region DNA rearrangements

V(D)J recombination is a multi-step process involving a complex of enzymes termed V(D)J recombinase as well as the products of two lymphoid specific genes RAG-1 and RAG-2 (recombination activating genes) and the enzyme terminal deoxynucleotidyl transferase (Tdt) (Alt et al., 1987). In brief V(D)J recombinases in conjunction with RAG proteins recognise the RSS and catalyse the formation of double strand breaks between both of the coding region/heptamer junctions. The DNA ends are held tightly in a complex by the RAG proteins and other associated DNA repair enzymes until the ligation of the coding sequences with each other and signal sequences with each other takes place. The two RSS are precisely joined to form the signal joint. Coding joint formation however, is more complex and contributes to antibody diversity. This junctional diversity at V-J and V-D-J coding joints is generated by a number of mechanisms including the removal of nucleotides (by exonuclease activity) and the addition of template encoded P-region nucleotides by DNA repair enzymes and, in the H chain only, non-template encoded N-region nucleotides (by TdT) (Alt et al., 1987).

As well as contributing to antibody diversity, imprecision in the joining of signal sequences may give rise to non-productive rearrangements whereby the triplet reading frame for translation is disrupted. The resulting VJ or VDJ unit will contain stop codons which interrupt translation. Gene segments which are joined in phase maintain triplet reading frames thus leading to productive rearrangements of VJ or VDJ units that can be translated into a complete region polypeptide. If in-phase rearrangements for both H and L chains are not produced the B cell will die by apoptosis (Schatz et al., 1992). Even though B cells are diploid they will only express the rearranged H and L chain genes from one chromosome. This process is called allelic exclusion and ensures that functional B cells never contain more than one functional $V_HD_HJ_H$ and one V_LJ_L. The recombination machinery is thought to be turned off once productive $V_HD_HJ_H$ and V_LJ_L units have been produced to ensure that the H and L chain genes on the homologous chromosomes are not expressed.

One model to account for allelic exclusion suggests that once a productive rearrangement is attained its encoded protein is expressed and acts as a signal for the recombination machinery to be turned off (Alt et al., 1987). The presence of the H chain signals for H chain recombination to stop and for κ chain recombination to start. If a productive κ rearrangement is made, a κ chain is produced and serves to switch off L chain recombination. If the κ rearrangement is non-productive for both κ alleles, rearrangement of the λ chain begins. If neither λ allele rearranges productively, the B cell dies by apoptosis (Alt et al., 1987). Some B cells which have made productive H and L chain rearrangements will nevertheless rearrange a second light chain locus or make a second rearrangement of the active light chain locus such that the previously productive genes are deleted (Prak and Weigert, 1995). This process is called receptor editing (the receptors being the surface IgG molecules on the B cell surface which bind to antigen) and allows a B cell to quickly change the sequence hence binding properties of an antibody. The H chain of an antibody may be replaced by a process analogous to receptor editing called receptor revision (Nemazee and Weigert, 2000).

After antigenic stimulation of a B cell, the H chain DNA can undergo a further process of rearrangement in which the $V_HD_HJ_H$ unit can combine with any C_H gene segment. The C_H gene segments are organised into a series of coding exons and non-coding introns. Each exon encodes a separate domain of the H chain constant region. Conservation of important biological effector functions is maintained by the limited numbers of C_H gene segments. The germline gene segments are arranged in order μ, δ, γ, ε and α. In an antibody the Cμ is the first constant region gene to be expressed (as IgM) but can be replaced by class switching to downstream constant regions (e.g. IgG, IgD, IgE or IgA) (Goldsby et al., 2000). This phenomenon allows clonally derived B cells to maintain the same V region specificity in association with different H chain constant regions specifying different effector functions (Alt et al., 1987).

1.7.3 The generation of diversity in immunoglobulins

The availability of a relatively large number of potentially functional variable region genes for recombination contributes to the diversity of possible antibody sequences (and therefore possible antigen binding sites) that can be produced. Diversity is amplified by imprecision at the joining of V_L-J_L, V_H-D_H and D_H-J_H segments with deletion or insertion of nucleotides (Tonegawa, 1983). After rearrangement of V_H, D_H and J_H genes to produce a functional heavy chain and of either V_κ and J_κ or V_λ and J_λ genes to produce a functional light chain, a B lymphocyte begins to secrete IgM antibody. By entering a germinal centre and recruiting T cell help, the B cell may then alter the isotype of the antibody produced by class switching and alter the sequence of the V_H and V_L domains by somatic hypermutation.

1.7.4 Rearranged V genes are further diversified by somatic mutation

During an immune response, B cells migrate to the germinal centres where antigen is presented to them in the presence of Th cells. Presentation of antigen specific to a B cell receptor will lead to proliferation (clonal expansion) and differentiation into plasma cells and memory B cells. Within the germinal centres B cells undergo somatic hypermutation, which leads to the alteration of a rearranged V_H or V_L sequence by introduction of nucleotide changes during the lifetime of a B cell which are subsequently passed on to all clonal descendants of that B cell. This process operates only in B lymphocytes and has been shown to be largely confined to the V domain, rarely being found in the C domain (Berek and Milstein, 1988; Storb, 1996). The observed mutation rate in these V regions is 10^{-3} to 10^{-4} per nucleotide per cell division.

Each mutation may be either silent ((S) leading to no change in amino acid sequence) or replacement ((R) leading to a change in amino acid sequence). R mutations may increase, decrease or fail to alter affinity of the antibody for antigen. Those somatic mutations that increase this affinity play a key role in the production of the secondary repertoire. B cells expressing higher affinity IgG on their surface will be selected by limited amounts of antigen and stimulated to divide faster hence leading to clonal expansion of these somatically mutated B cells (Wagner and Neuberger, 1996). The presence of antigen leads to a selective pressure that favours the accumulation of particular R mutations in the CDRs, because these are the areas of the sequence where such mutations can exert the greatest positive effects on binding affinity (Shlomchik et al., 1987). Thus R:S ratios tend to be higher in CDRs than in FRs for antibodies which have been subject to this antigen driven process (Shlomchik et al., 1987).

A number of authors have noted, however, that codon usage is different in CDRs and FRs and this means that the inherent tendency for a mutation to be R rather than S is higher in CDRs (Chang and Casali, 1994). Statistical methods have been designed which enable the distribution of R and S mutations to be analysed in a way which allows for this inherent difference between CDRs and FRs. The binomial method of Chang and Casali (1994) has been used widely, but has been superseded by a more accurate cumulative multinomial method. This new statistical method (Lossos et al., 2000) is more accurate since, unlike the binomial method, it takes into account all four possible outcomes of a particular mutation i.e. R and S mutations in the CDRs and/or FRs of the gene. Calculations using the multinomial method can be made using the JAVA applet provided by Lossos *et al.* at http://www-stat.stanford.edu/immunoglobin. For instance, a low p value ($p<0.05$) obtained by the multinomial method suggests that the observed distribution of R and S mutations is very unlikely to have arisen by chance alone and is likely to have arisen as a result of antigen selection of mutations. In practice antigen drive favours an excess of R mutations in the CDRs but an excess of S mutations and scarcity of R mutations in FRs. Therefore low p values may be obtained for FRs as well as CDRs.

The mutations introduced by somatic hypermutation of variable region sequences are predominantly single nucleotide substitutions (Ignatovich et al., 1997).Somatic insertions and deletions occur to a lesser degree and would appear to be closely linked to nucleotide substitutions and probably created by the same enzymatic machinery (de Wildt et al., 1999b). Somatic mutations are limited to the V region of the Ig gene and its proximate flanks covering a variable stretch of 1-2 Kb from the 5` end of the transcribed region (Storb, 1996). Activation of V region mutations generally requires T cell help, is restricted to a brief period in B cell development and targeted to specific residues (hot spots) with characteristic motifs such as RGYW (R = purine, Y = pyrimidine, W = A/T). Such motifs have an intrinsic mutability which does not depend upon antigen selection, have no strand bias and are concentrated within the CDR`s (Dorner et al., 1998). Somatic mutations spread diversity to regions at the periphery of the antigen binding site that are conserved in the primary repertoire so the pattern of sequence diversity of the mature (secondary) repertoire is more evenly distributed across the entire antigen binding site (Tomlinson et al., 1996a).

The precise molecular mechanism responsible for somatic hypermutation is not fully understood. In order to undergo somatic mutation the Ig gene must be transcriptionally active ((Peters and Storb, 1996; Fukita *et al.*, 1998; Neuberger *et al.*, 1998; Storb, 1998). The DNA mismatch repair system, as well as a newly described DNA polymerase (pol μ), has been implicated in somatic mutation (Cascalho *et al.*, 1998; Wiesendanger *et al.*, 1998; Dominguez *et al.*, 2000). More recently a novel cytidine deaminase, expressed only in germinal centre B cells, has been shown to play a crucial role in both somatic mutation and class switch recombination (Muramatsu et al., 2000; Revy et al., 2000). DNA double strand breaks have also been proposed as the initiating event and thence the focus for error prone DNA repair in creating somatic mutations (Papavasiliou and Schatz, 2000).

1.7.5 Human V region gene repertoire

By comparing the V_H and V_L sequences of an antibody with those of the germline genes from which they were derived, the positions and nature (R or S) of the somatic mutations can be identified. To do this accurately, one must be confident that the assignment of germline genes is correct. Such confident assignment is now feasible for human antibodies, because all the genes in the human heavy, κ and λ loci have been identified and mapped (Matsuda *et al.*, 1993; Schable and Zachau, 1993; Cook *et al.*, 1994; Williams *et al.*, 1996). Furthermore, although allelic polymorphism occurs in some V_H, V_κ and V_λ genes, it can be excluded as a cause for most differences between antibody sequences and germline genes, because it is possible to use specialist software to screen databases of all known alleles to find the closest possible germline match for any antibody. It is unlikely that large numbers of undiscovered alleles exist. New polymorphisms have been reported for only approximately 15% of all V_H, V_κ and V_λ genes over the past five years and most of these are single nucleotide changes only (Tomlinson personal communication).

1.7.6 Genetics of the Ig V germline gene repertoire

In humans, functional immunoglobulin heavy, kappa, and lambda genes are found on chromosomes 14, 2 and 22 respectively. All functional V genes have now been mapped to these three chromosomes (Schable and Zachau, 1993; Cook *et al.*, 1994; Williams *et al.*, 1996).

1.7.7 Human heavy chain Ig germline gene locus

Each heavy chain locus contains approximately 50 functional V_H (Matsuda *et al.*, 1993; Cook *et al.*, 1994), 30 D_H (Corbett et al., 1997) and 6 J_H genes (Ravetch et al., 1981). The genes in each locus are grouped in families on the basis of sequence homology.

1.7.7.1 Human V_H locus

Analysis of the human Ig V_H locus at chromosome 14q23.3 was completed in 1994 (Cook et al., 1994). The human germline V_H repertoire consists of approximately 50 functional V_H gene segments spanning 1100kb upstream of the J_H segments. By definition, functional V_H segments must contain an open reading frame (ORF) and must have been observed in functional VDJ rearrangements. They are interspersed among an equivalent number of pseudogenes (Tomlinson et al., 1992), which are nucleotide sequences that are stable components of the genome but are incapable of being expressed. Human V_H segments can be divided into seven families, V_H 1-7 on the basis of sequence homology, which are interspersed throughout the locus. Each family member shares 80% homology at the nucleotide sequence level and some families are more highly related (V_H4) than others, which are more divergent (V_H5). Some V_H families are larger than others, with the largest contribution made by the V_H3, V_H4 and V_H1 families (Cook and Tomlinson, 1995).

Twenty four human V_H segments have also been mapped to chromosomes 15 and 16. However, since these chromosomes lack D_H, J_H and C_H segments they are unlikely to be functional. Expression of V_H segments on chromosomes 15 and 16 would require interchromosomal recombination, which has not been demonstrated in B cells (Matsuda et al., 1993).

The exact dimensions of the V_H locus and number of functional segments vary between individuals. Two types of polymorphism affect the germline repertoire: the insertion or deletion of V_H segments and the occurrence of different alleles of the same segment. Allelic polymorphism is limited. Insertion/deletion polymorphism however, is fairly common and 75% of people have an extra group of genes located between segments 3-30 and 4-31 which are listed in VBASE, a sequence directory containing the complete human Ig gene repertoire that is constantly being updated with new alleles (Tomlinson et al., 1996b).

1.7.7.2 Human D_H locus

The complete nucleotide sequence of the human Ig D segment locus on chromosome 14q32.3 was determined by Corbett *et al.* (1997). They identified a total of 27 D segments which could be grouped into seven families based on sequence homology. Previously, conventional VDJ recombination alone had not been thought sufficient to explain the extreme diversity found in V_HCDR3. Various hypotheses had been proposed to explain this extensive versatility such as the DD recombination (regardless of the 12/23 rule) (Sanz, 1991), occurrence of DIR segments (longer sequences interspersed amongst the functional D_H segments) (Ichihara et al., 1988), inverted D segments, or the use of minor D segments found on chromosome 15 (Cook and Tomlinson, 1995). A systematic analysis however to compare the complete sequence of the human Ig D segment locus with a database of rearranged sequences to identify all the germline D segments and any other sequences that contribute to the extreme diversity of V_HCDR3 found no evidence to suggest that any of the above mechanisms took place (Corbett et al., 1997). The authors felt that conventional VDJ recombination which obeys the 12/23 rule was sufficient to explain the wealth of lengths and sequences of V_HCDR3. The fact that they found no sequences that matched DIR genes or DD fusions may have arisen due to the stringent criteria applied whereby at least ten consecutive nucleotides of identity were generally required to confidently assign a D segment to its germline match, depending upon the length of CDR3. Previous studies have taken only five nucleotides of identity with one mismatch as their minimum cut off for identifying a germline sequence (Brezinschek et al., 1995).

1.7.7.3 Human J_H locus

The human J_H locus contains nine J-like gene segments, six of which appear to be functional and account for most of the known human H chain amino acid sequences whilst three are pseudogenes (Ravetch et al., 1981).

1.7.8 Human light chain immunoglobulin germline genes

1.7.8.1 Human kappa locus

The kappa locus has been mapped to chromosome 2p11-12 and contains approximately 40 functional V_κ (Schable and Zachau, 1993) and five J_κ genes with no D gene segments (Schable and Zachau, 1993). There are seven V_κ families, the members of which are interspersed throughout the locus amongst 36 pseudogenes. Additional V_κ genes are located upon chromosomes 1 and 22 but these are pseudogenes. Allelic polymorphism is rare and where present, alleles differ by only one or two nucleotides (Schable and Zachau, 1993). There is only one C_κ gene segment so no alternative isotypes of the κ chain exist.

1.7.8.2 Human lambda locus

Sequence analysis of the human lambda locus on chromosome 22q11.2 has identified 30 functional V_λ (Williams et al., 1996) and four functional J_λ genes (Vasicek and Leder, 1990). Ten V_λ families exist, spread amongst three distinct V_λ gene clusters. Some families are larger than others and the larger $V_\lambda 1$, 2 and 3 families are more commonly expressed in humans. Similarly to the human V_H and V_κ loci, allelic polymorphism is relatively rare and where present, alleles differ by only one or two nucleotides (Williams et al., 1996).

The C_λ locus, also located upon chromosome 22, contains seven different genes of which four are functional and three are pseudogenes. As a result λ chains like heavy chains have several isotypes. Each of the four functional C_λ genes is preceded by a single J_λ gene segment of which $J_\lambda 2$ and $J_\lambda 3$ are identical therefore mAb sequences can include only one of three different J_λ sequences (Vasicek and Leder, 1990).

1.8 SEQUENCE ANALYSIS OF MONOCLONAL ANTIBODIES

1.8.1 Interpretation of antibody sequences analysis

An understanding of the genetic origin of Ig sequences as determined in the previous sections, enable us to analyse the sequence of any antibody molecules by asking the following questions. Which were the genes of origin? What is the extent of somatic hypermutation and is it likely to have been driven by antigen? Are there sequence motifs common to antibodies which share particular binding properties? Do these sequence motifs correspond with sites of somatic hypermutation and with the predicted contact sites of the antibody?

Which were the genes of origin? In analysing the sequences of the V_H and V_L domains of a particular antibody, therefore, it is possible to deduce which of the available genes in the repertoire have been used to encode these domains and the extent to which somatic mutations and modification at the junctions has occurred. By repeating this analysis for a large number of different Ab such as aPL, it would theoretically be possible to deduce whether particular genes are preferentially rearranged to create PL binding sites. To reach this conclusion, however, it is vital to recognise that each locus shows an intrinsic bias towards the rearrangement of certain genes. In other words, some $V_{H,}$ V_κ and V_λ genes are more likely to be used to encode antibodies than others, regardless of the specificities of the antibodies produced. This bias is shaped by intrinsic genetic factors as it is seen in both productive and non-productive gene rearrangements (Brezinschek *et al.*, 1997; Ignatovich *et al.*, 1997; Ignatovich *et al.*, 1999). Measurement of the frequency of expression of different V_H/V_L pairs has demonstrated that preferential pairing of specific genes or families does not occur. The most likely finding in a single B cell is the expression of a commonly rearranged V_H gene together with a commonly rearranged V_L gene (Brezinschek et al., 1998; de Wildt et al., 1999a). The pattern of V_H/V_L gene usage and pairing in peripheral blood lymphocytes (PBL) is not significantly different in patients with SLE compared to healthy people (de Wildt et al., 2000). Investigations of V_H/V_L pairing in patients with primary APS have not been reported.

What is the extent of somatic hypermutation and is it likely to have been driven by antigen? Having identified the positions of R and S mutations within the V_H and V_L sequences, it is possible to analyse the distribution of these mutations using the mulitinomial method (Lossos et al., 2000).

Are there sequence motifs common to antibodies which share particular binding properties? In antibodies which seem likely to have been modified by antigen driven selection of mutations, it is possible to identify particular sequence features which result from these modifications and which may therefore enhance binding to the driving antigen.

Do these sequence motifs correspond with sites of somatic hypermutation and with the predicted contact sites of the antibody? Analysis of the known crystal structures of 26 different Ag-Ab complexes has shown that certain residues within CDRs of the H and L chains are more likely than others to contact antigens (MacCallum et al., 1996). For example, antigens are much more likely to contact residues towards the beginning of heavy chain CDR2 than those towards the end of that CDR. These authors suggested that analyses of CDR AA composition could be much more informative when the likelihood that an AA at a particular position will be involved in antigen contact is taken into account.

1.8.2 Sequence analysis of anti-DNA antibodies

Sequence analysis of the antigen combining sites of monoclonal Ab serves as a powerful tool to gain greater understanding of features at the molecular level which serve to distinguish pathogenic from non-pathogenic Ab. This form of analysis has been applied to sequences of both murine and human anti-DNA Ab. An extensive review of the sequences of over 300 murine monoclonal anti-DNA Abs from many different models of SLE, revealed that certain V_H and V_κ genes were used preferentially, and some pairings of these genes were more common than others (Radic and Weigert, 1994).

The antigen driven accumulation of somatic mutations has also been found to be important in determining the ability of murine anti-DNA Ab to bind dsDNA. Marion *et al.* (1992) analysed the sequences of 117 monoclonal anti-DNA antibodies from 11 different (NZB x NZW)F_1 mice. In some cases, IgG produced by a single mouse were derived from the same expanded B cell clone whereas the majority of IgM were not. Clonally related IgM and IgG hybridomas were obtained from two individual mice. Within a single clone, more mutations were found in the IgG than the IgM, particularly in the CDRs. This increase in mutations was associated with increased ability to bind dsDNA (Marion et al., 1992).

Somatic mutations creating arginine (Arg), lysine (Lys) and asparagine (Asn) residues in the CDRs were also found to occur very commonly by Radic and Weigert (1994). All of these amino acids may promote binding to the negatively charged DNA double helix by charge interactions (Arg and Lys) or hydrogen bond formation (Asn). Computer modelling programs, which predict the three dimensional structures of antibodies from their amino acid sequences (Martin *et al.*, 1991; Chothia *et al.*, 1998), have been used to predict sites of contact between the dsDNA molecule and arginine residues in the CDRs (Radic and Weigert, 1994). These predictions were subsequently tested by altering Arg residues at those sites by mutagenesis and demonstrating that this altered DNA-binding affinity in the way predicted by the model (Radic et al., 1993).

In conclusion, sequence analysis of murine monoclonal anti-DNA antibodies has shown that there is preferential use of V_H and V_κ genes to encode these antibodies and that there is antigen-driven accumulation of somatic mutations in the CDRs leading to the prevalence of certain amino acid residues, in particular Arg, in the CDRs.

Human monoclonal Ab are difficult to produce, hence far fewer human than murine monoclonal Ab have been sequenced. Despite the relative lack of human data it is important to discover whether the conclusions derived from studying murine Ab are equally applicable to human Ab for a number of reasons. Firstly there are major differences between the available Ig gene repertoires in humans and mice. For instance, there are only about 50 functional human V_H genes (Cook et al., 1994) and up to about 1000 in the mouse (Kofler et al., 1992). Secondly, because the entire human V region gene repertoire has been characterised, it is possible to identify sites and types of somatic mutation with an accuracy that may not be possible for all murine Ab. Lastly, approximately 40% of human Ab but only 5% of murine Ab carry λ chains which means it is impossible to make deductions about utilisation and mutation of λ genes in anti-DNA Ab found in SLE by studying murine Ab.

A recent review (Rahman et al., 2002a), identified published reports of 66 human anti-DNA Ab which included sequence data. Systematic analysis of the human sequences found no evidence for a preferential usage of particular human V_H or V_L genes. Somatic mutations commonly occurred in IgG and IgA Ab and were clustered in the CDRs. Statistical analysis using the method of Lossos *et al.* (2000) strongly supported the idea that antigen drive was responsible for this pattern of mutation in the majority of cases. Furthermore an accumulation of Arg, Asn and Lys at sites of antigen contact, as defined by MacCallum *et al.* (1996), were found in the IgG and IgA Ab largely as a result of somatic mutations. Similarly to murine Ab, computer modelling programs have been used to show that Arg residues in some human anti-DNA Ab are also important sites of contact with dsDNA (Kalsi et al., 1996; Rahman et al., 2001).

Overall, other than the differences in Ig gene utilisation, sequence analysis of both human and murine anti-dsDNA Ab has revealed similar findings. Therefore a large body of evidence now exists which suggests that the amino acids Arg, Lys and Asn are important in conferring the ability to bind dsDNA, especially where they are derived from antigen-driven accumulation of somatic mutations in the CDRs of IgG and IgA Abs. The same principles of sequence analysis may be applied to human and murine monoclonal aPL.

1.9 SEQUENCE ANALYSIS OF aPL

1.9.1 Previous evidence from sequence analysis of aPL

1.9.1.1 Animal Studies

Unlike murine anti-dsDNA Ab relatively little has been published on murine aPL. One reason for this is that the full range of clinical manifestations seen in the human syndrome is not found in any one animal model of APS. Thus far aPL have been produced from animal models which show consistent serological features of the syndrome but which generally show either the obstetric or thrombotic clinical features but not both (Radway-Bright et al., 1999). Studies of aPL derived from MRL lpr/lpr mice (Kita et al., 1993) and NZW x BXSB F1 mice (Kita et al., 1994; Monestier et al., 1996) do not reveal convincing evidence of any particular gene preference, somatic mutations or specific residues in CDRs which are important for binding. The most commonly expressed V_H genes in these murine aPL were members of the J558 murine V_H family, but almost 50% of all spontaneously activated B cells in MRL lpr/lpr mice express J558 genes regardless of antigen specificity (Foster et al., 1991). Despite some evidence of clonal expansion in development of aPL and some clustering of replacement mutations in V_H CDRs found in one study (Monestier et al., 1996), the extent to which antigen driven somatic mutations and the presence of certain residues in CDRs are important in murine aPL remains uncertain (Rahman et al., 1996).

1.9.1.2 Human Studies

Previous sequence analysis of human monoclonal aPL antibodies showed that IgG, but not IgM, aPL often contain large numbers of somatic mutations in their variable regions. In many cases R:S ratios were particularly high in the CDRs, particularly in IgG antibodies and those derived from patients with APS (Rahman et al., 1996). This pattern of mutation is characteristic of antigen driven clonal expansion to create high affinity antibodies (Shlomchik et al., 1987). These monoclonal aPL tended to have an accumulation of certain residues such as Arg and Asn in their CDRs, but the evidence for this was not as strong as for anti-DNA antibodies (Radic and Weigert, 1994; Isenberg *et al.*, 1997).

1.9.1.3 Conclusions and limitations of previous aPL sequence analysis

Conclusions from previous aPL sequence analysis postulated a link between specificity for negatively charged PL and the presence of accumulations of Arg or Asn residues in CDRs. In IgG (but not IgM) aPL these residues probably arose due to antigen driven somatic mutation (Rahman *et al.*, 1996; Rahman *et al.*, 1998). Limitations of these previous analyses include: the small numbers of published IgG aPL; no consideration of the relevance of ability to bind ß$_2$GPI; an incomplete knowledge of the human V gene repertoire; and less advanced statistical methods for analysing the pattern of mutations.

In recent years, more human IgG aPL have been derived from patients with APS (rather than those with SLE alone), many of these have been tested for ability to bind ß$_2$GPI, and the first sequences of human monoclonal IgG anti-ß$_2$GPI Ab fragments derived by phage display have been reported (Chukwuocha et al., 1999). Thus, more sequence information relating to clinically relevant antibodies is now available and it is now clear that a large proportion of pathogenic antibodies in APS probably bind epitopes on ß$_2$GPI. Furthermore, it is now possible to be more confident in predicting which residues within a particular antibody sequence are most likely to form close contacts with antigen (MacCallum et al., 1996). These predictions may be strengthened by the use of computer modelling programs to show the probable shape of the antigen binding site encoded by a particular antibody sequence. I therefore performed a new analysis of published human aPL and anti- ß$_2$GPI antibody sequences with colleagues (Giles et al., 2003b).

1.9.2 Updated aPL sequence analysis

All published human and murine monoclonal aPL and anti-ß$_2$GPI sequences were identified by a PubMed search using the keywords - monoclonal, antiphospholipid antibodies. The original papers describing the V region DNA sequences of these antibodies were obtained and the sequences analysed. Sequences were compared to all the alleles of human Ig genes in the VBASE database using DNAPLOT software. Both the alignment program and the database are available at http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html. Any differences between the germline and expressed antibody sequences were considered to be somatic mutations unless they were at sites of junctional diversity or could be shown to arise from PCR primers used to clone the antibody cDNA in the original experiment.

Statistical analysis, using the multinomial method (Lossos et al., 2000), of the distribution of R and S mutations was carried out for all the V_H or V_L sequences where homology to the germline gene was less than 98%. It was assumed that sequences with homologies greater than this contained too few mutations for statistical analysis to be informative (Schroeder and Dighiero, 1994). The sequences of the monoclonal antibodies were aligned and the amino acid residues most likely to contact antigen were determined according to the paper of MacCallum *et al.* (1996)

1.9.3 Results of aPL sequence analysis

Thirty six human aPL were identified, 22 of these were of IgM isotype and were derived from lymphocytes immortalized by Epstein Barr transformation or by fusion with immortal cells to form hybridomas. The genes of origin, homology values and binding characteristics of these antibodies are shown in Tables 1.6 and 1.7.

As noted previously, these IgM antibodies can be divided into two groups (Rahman *et al.*, 1996; Rahman *et al.*, 1998). These are shown as groups A and B in Table 1.6. The first group (A) of 15 antibodies are polyreactive and were not selected for specific binding to PL or for relevance to APS (Dersimonian *et al.*, 1987; Hoch and Schwaber, 1987; Cairns *et al.*, 1989; Logtenberg *et al.*, 1989; Siminovitch *et al.*, 1989; Hohmann *et al.*, 1995; Rioux *et al.*, 1995). Most of these mAb were actually selected for binding to DNA and were incidentally found to bind PL (often with low affinity) on panel Ag testing. Most of the antibodies in group A were derived from PBL of healthy individuals. Only four (18/2, 1/17, C119 and C471) of the 15 aPL identified in this group were derived from patients with SLE, none of whom had features of the APS. Binding to β_2GPI was not reported for any of these Ab.

References for mAb Tables 1.6 and 1.7

Table 1.6

1) Dersimonian *et al.* (1987)
2) Hoch *et al.* (1987)
3) Siminovitch *et al.* (1989)
4) Cairns *et al.* (1989)
5) Logtenberg *et al.* (1989)
6) Rioux *et al.* (1995)
7) Homann *et al.* (1995)
8) Siminovitch *et al.* (1990)
9) Mariette *et al.* (1993)
10) Harmer *et al.* (1995)
11) Denomme *et al.* (1994)
12) Demaison *et al.* (1995)
13) Lai *et al.* (1998)

Table 1.7

1) Van Es *et al.* (1992)
2) Menon *et al.* (1997)
3) Ikematsu *et al.* (1998)
4) Lai *et al.* (1998)
5) von Landenberg *et al.* (1999)
6) Chukwuocha *et al.* (1999)

Abbreviations for Tables 1.6 and 1.7

The symbols † denote binding to $ß_2$GPI and * lupus anticoagulant activity. Abbreviations: - NM – No Match; NP – Not Published; SLE – Systemic Lupus Erythematosus; PBL – Peripheral Blood Lymphocyte; PD – Phage display; CLL – Chronic Lymphocytic Leukaemia; ss/ds DNA – single/double stranded DNA; Poly I – Polyinosinic acid; Poly G – Polyguanolic acid; Poly U – Polyuracil; Poly dA-dT – Polydeoxyadenylate-thymidylate sodium salt; Poly dG-Poly dC – Polydeoxyguanylate-polydeoxycytidylate; Plt – Platelet; CL – Cardiolipin; Hel – Hen egg lysozyme; Cyt c - Cytochrome c; DOPE –dioleoylphosphatidylethanolamine; RF – Rheumatoid Factor; PT – Prothrombin; OVA – Chicken ovalbumin; BSA – Bovine Serum Albumin.

In Table 1.7 only binding to antigens other than anionic PL is indicated.

mAb	origin	V_H gene	Nucleotide Homology	D_H	J_H	V_L family	V_L gene	Nucleotide Homology	J_L	Binding	Reference
Group A											
m18/2	SLE PBL	V3-23	100	NM	NM	NP	NP	NP	NP	ssDNA, poly-I, poly-dT, Plt	1
m1/17	SLE PBL	V3-23	100	NM	NM	NP	NP	NP	NP	ssDNA, Plt, Vimentin	1
C6B2	Sickle cell spleen	V4-61	97.5	D3-10	J_H3b	NP	NP	NP	NP	Poly-G/I/U, ss/dsDNA Poly-dAdT-PolydAdT PA, CL, PI, PG	2
Kim4.6	Healthy Tonsil	V3-30	100	D3-10	J_H6a	V_λ1	1b	100	NM	ss/dsDNA, poly-dAdT poly-dG-Poly-dC, RNA, CL	3,4
A10	Healthy PBL	V6-01	99	NM	J_H6a	NP	NP	NP	NP	ss/dsDNA, CL, PdT, Hel	5
A431	Healthy PBL	V6-01	98	NM	J_H6a	NP	NP	NP	NP	ss/dsDNA, CL, PdT, Cyt c	5
L16	Fetal Liver	V6-01	100	NM	J_H6a	NP	NP	NP	NP	ssDNA, CL, PdT	5
C119 *	SLE, PBL	V3-23	95.5	NM	NM	V_κ3	L6	100	J_κ4	ssDNA, Plt, CL, DOPE	6
B122	Healthy PBL	V1-18	97	NM	NM	V_κ1	L12a	98.3	NM	ssDNA, Plt, CL, DOPE	6
B6204	Healthy PBL	V3-23	97	NM	NM	V_κ3	A27	99.6	NM	ssDNA, Plt, CL, DOPE, RF	6
C471	SLE, PBL	V3-64	99.6	NM	NM	V_κ3	L2	100	J_κ2	ssDNA, Plt, DOPE	6
H3	Healthy PBL	V1-46	97	D6-13	J_H4	V_κ3	3l	97	J_λ2/3a	CL, PS, PE, DT, TT	7
H5	Healthy PBL	V4-30	96	DIRL	J_H 4	NP	NP	NP	NP	CL, DT, TT	7
A5	Healthy PBL	V3-23	100	DHF16	J_H 6	V_λ3	3p	97	J_λ2/3a	CL, DT, TT, ssDNA	7
Bou 53.6	Healthy Tonsil	V3-23	100	D4	J_H 6	V_κ1	L12	99	J_κ2	CL, polyreactive	7
Group B											
Kim 13.1	Healthy Tonsil	1-e	100	NM	J_H 5b	V_κ3	L6	100	J_κ4	CL, RF	8
REN †	CLL	V4-61	100	NM	J_H 3b	V_λ8	8a	99.7	J_λ3b	CL, ssDNA	9
BH1 *†	PAPS PBL	V3-30	100	NM	NM	V_λ3	3r	97.5	J_λ3b	anionic PL	10
STO 103	Healthy Tonsil	V4-61	100	D3-03	J_H 4a	V_κIV	B3	100	J_κ2	Plt, anionic PL	11
RSP 4	SLE PBL	V3-30	96	D4-23	J_H 4b	V_λ1	le	98.3	J_λ1	CL	12
B9421 *	SLE/APS PBL	V3-72	100	D21-9	J_H 3	V_λ2	2a2	99.3	J_λ3b	LA	13
B9427 *†	SLE/APS PBL	V3-15	100	D21-9	J_H 4	V_κ2	A17	100	J_κ3	LA	13

Table 1.6 Sequence Features of Polyreactive IgM, group A and More Specific IgM, group B aPL

mAb	origin	VH gene	Nucleotide Homology	D_H	J_H	VL family	VL gene	Nucleotide Homology	J_L	Binding to other Ag	Reference
R149	SLE PBL	V1-69	96.6	NM	J_H6a	$V_\kappa 2$	A19	99.3	$J_\kappa 2$	ssDNA	1
LJ-1	SLE PBL	NP	NP	NP	NP	$V_\lambda 3$	3h	98.6	$J_\lambda 2/J_\lambda 3a$	histone	2
AH-2	SLE PBL	V5-51	94.9	D4-23	J_H 6b	$V_\lambda 1$	le	99	$J_\lambda 3b$	no	2
DA-3	SLE PBL	V5-51	95.2	D3-09	J_H 6a	$V_\lambda 1$	le	98.3	$J_\lambda 3b$	histone	2
UK-4	SLE PBL	V3-74	95	NM	J_H 4b	$V_\lambda 2$	2a2	94	$J_\lambda 2/J_\lambda 3a$	No	2
516	PAPS PBL	V1-69	97.3	D3-10	J_H 6b	$V_\kappa 1$	L19	96.9	$J_\kappa 4$	ssDNA, recombinant human insulin, tetanus toxoid, human thyroglobulin, BSA	3
519	Healthy PBL	V4-34	97.9	NM	J_H 5a	$V_\kappa 3$	L2	98.9	$J_\kappa 4$	No	3
B9416 *	SLE/APS PBL	V3-23	98	NM	J_H 4b	$V_\kappa 1$	012/02	98.6	$J_\kappa 1$	no	4
HL-5B *	PAPS PBL	V3-53	89	NM	NM	NP	NP	NP	NP	oxLDL	5
RR-7F	SLE PBL	V3-24	86.1	NM	NM	NP	NP	NP	NP	oxLDL	5
B14 †	Healthy PD	V3-23	91.9	D2-15	J_H 4b	$V_\kappa 1$	A20	97.6	$J_\kappa 2$	no	6
B22 †	Healthy PD	V6-01	93.7	NM	J_H 4b	$V_\kappa 1$	A20	98.1	$J_\kappa 3$	no	6
B27 †	Healthy PD	V6-01	93.7	NM	J_H 4b	$V_\kappa 1$	L15	95.8	$J_\kappa 4$	no	6
P11 †	Healthy PD	V4-30	92.7	D3-03	J_H 4b	$V_\kappa 1$	L8	96	$J_\kappa 3$	PT, OVA, ssDNA	6

Table 1.7 Sequence Features of IgG aPL

Although the antibodies in Group A seem likely to have limited relevance to the pathogenesis of APS, they are included in this analysis to ensure a comprehensive analysis of all human aPL sequences. The group A antibodies are likely to be representative of non-pathogenic natural autoantibodies and thus their sequence characteristics serve as a useful comparison with those of the more specific and probably more pathogenic IgM aPL in group B and of the IgG aPL.

Members of the second group B of seven IgM antibodies are likely to be more relevant to the pathogenesis of APS (Siminovitch *et al.*, 1990; Mariette *et al.*, 1993; Denomme *et al.*, 1994; Demaison *et al.*, 1995; Harmer *et al.*, 1995; Lai *et al.*, 1998). Most of these antibodies showed specific binding to PL alone despite testing against a range of other antigens. The exception was REN, which also binds ssDNA. Three antibodies in group B showed LA activity (BH1, B9421 and B9427). B9427 bound ß$_2$GPI, REN showed ß$_2$GPI dependent binding to PL and BH1 showed serum dependent binding to PL (the effect of ß$_2$GPI was not tested formally). Three antibodies (BH1, B9427 and B9421) were produced from the lymphocytes of APS patients and another one (RSP4,) from a patient with SLE and serum aPL, who did not have clinical features of APS.

The other 14 aPL identified were IgG antibodies.(Van Es *et al.*, 1992; Menon *et al.*, 1997; Ikematsu *et al.*, 1998; Chukwuocha *et al.*, 1999; von Landenberg *et al.*, 1999) The genes of origin, homology values and binding characteristics of these antibodies are shown in Table 1.7. Ten of these IgG antibodies were derived from immortalised lymphocytes and four (P11, B22, B27 and B14) by repertoire cloning using the phage display method (Chukwuocha et al., 1999). These four antibodies were produced from cDNA derived from lymphocytes of a patient who had circulating anti-ß$_2$GPI antibodies, although he did not have APS. The cDNA was cloned into phage display vectors which allowed the production of a library of phages carrying surface Fab derived from heavy and light chain sequences expressed in that patient's lymphocytes although the V_H/V_L gene combinations are artificial and not all may have been expressed in patient serum. Phages carrying anti-ß$_2$GPI Fab were selected by binding to plates carrying ß$_2$GPI. Thus the heavy and light chain gene combinations of the aPL selected by phage display may not be representative of naturally occurring aPL. Such a discrepancy does not occur in monoclonal aPL that are derived from immortalised B cells in which the V_H /V_L gene combination is definitely expressed by at least one B cell in the individual from which it was produced.

Of the other 10 IgG aPL, seven were derived from patients with SLE (one of whom had APS) whilst two were produced from patients with PAPS. Most of the monoclonal IgG aPL showed specificity for PL or β_2GPI, as shown in Table 1.7. The only monoclonal aPL shown to be pathogenic in the papers studied were the IgG aPL 516 and 519 derived from a PAPS patient and healthy subject respectively. Both of these monoclonal aPL induced a significantly higher rate of fetal resorption and a significant reduction in fetal and placental weight following intravenous injection into mated BALB/c mice (Ikematsu *et al.,* 1998).

1.9.4 Which were the genes of Origin?

The frequency of use of V, D and J genes to encode monoclonal aPL was compared with that which would be expected from the intrinsic bias of the Ig gene recombination process.

1.9.4.1 Heavy chain genes

Twelve of the 15 polyreactive aPL in group A, all seven of the more specific IgM aPL in group B and 9 of 13 published IgG aPL used genes from the V_H 1,3, or 4 families. These are the largest and most commonly expressed V_H families in both normal donors and patients with SLE (de Wildt et al., 2000). The other three polyreactive antibodies used the V_H 6 gene V6-01, but were derived from an experiment which set out specifically to produce monoclonal Ab encoded by that gene (Logtenberg et al., 1989). The apparently high prevalence of polyreactive aPL encoded by V6-01 is therefore misleading.

The most commonly used gene amongst polyreactive IgM aPL and IgG aPL is V3-23 which is the most commonly rearranged V_H gene in IgM secreting B cells regardless of Ag specificity (Brezinschek et al., 1997). A group of V_H genes that are particularly likely to be rearranged due to the intrinsic bias in the recombination process has been identified by single cell PCR in peripheral B cells (Brezinschek et al., 1997; de Wildt et al., 2000). This group includes V3-23, V3-30, V3-07, V4-34, V4-39, V4-59, V1-18, V1-69 and V5-51. Members of this group of genes encode V_H sequences in 8 of the 15 polyreactive IgM antibodies, 7 of the 13 published IgG aPL, but only two of the seven more specific IgM antibodies.

For a number of the antibodies studied, it was not possible to match the heavy chain CDR3 sequence to any known D_H gene. The reason for this is that the DNAPLOT software used very stringent criteria, described in section 1.7.7.2, for aligning Ab sequences to germline D segments (Corbett et al., 1997). Six of the 15 IgM sequences in group A, four of the seven in group B and five of the 13 IgG sequences could be matched to known D genes. The majority of the D segments matched were from the D2 and D3 families which have previously been shown to dominate the expressed repertoire (Brezinschek et al., 1997; Corbett et al., 1997; de Wildt et al., 2000).

J_H genes of origin could be identified for nine IgM antibodies in group A, six in group B and 11 IgG antibodies. Where no match could be made, this was usually because the published sequence did not extend as far as the J region. The majority of the sequences matched the J_H3, J_H4 or J_H6 genes, which are the most commonly rearranged J_H genes in peripheral B cells (de Wildt et al., 2000).

1.9.4.2 Light chain genes

Information from single cell PCR experiments (Brezinschek et al., 1998) and from analysis of human V_L cDNA libraries (Ignatovich et al., 1997) has allowed the identification of V_K and $V\lambda$ genes which account for most of the expressed repertoire. Of the light chain sequences of these aPL, all polyreactive IgM, all IgG and five of seven more specific IgM aPL used genes which are members of the three largest and most commonly expressed V_K (I-III) or $V\lambda$ (1-3) families. Six of the 8 published V_L sequences of IgM in Group A, six of seven sequences in Group B and 9 of 12 published IgG aPL sequences utilised V_K or $V\lambda$ genes which are known to be commonly rearranged and expressed (Cox et al., 1994; Ignatovich et al., 1997). The most commonly rearranged V_K genes include A3, A17, A20, 02/12, L8, L12, L6, A27, L2, and B3 while commonly rearranged V_λ genes include 1b,1c,1e, 2a2,2b2,2c, 3h and 3r.

In the cases where assignment to a J_L gene could be made, it was found that λ encoded sequences always used $J\lambda2$ or $J\lambda3$ genes, whereas the κ encoded sequences were split evenly between J_K2, J_K3 and J_K4. There was no difference between IgM and IgG antibodies. These results mirror the known usage of these genes in peripheral lymphocytes (Dorner *et al.*, 1999; de Wildt *et al.*, 2000), though the low rate of use of the $J_{\kappa}1$ gene amongst aPL is a little surprising.

In summary, therefore, the pattern of gene usage in these aPL antibodies is generally that which would be expected from the intrinsic bias of the recombination mechanism. Genes that are commonly rearranged by that mechanism are also commonly used to encode both IgM and IgG aPL. The only exception is in the group of more specific IgM aPL, where five of seven antibodies use V_H genes which are not commonly rearranged in other antibodies. These genes include V1-e, V3-15, V3-72 and V4-61, which are not used to encode either polyreactive IgM aPL or IgG aPL. Each of these genes is used in its germline configuration. One might therefore suspect that the germline sequences of these genes could contain motifs which were beneficial in binding to PL or β_2GP1. However, on comparing the sequences of these germline genes, no common motif in either CDR1 or CDR2 is evident. The genes are derived from three different families (V_H 1, V_H 3 and V_H 4) and each has a different three dimensional fold structure (predictable from the amino acid sequence). Therefore, there is no evidence to support the idea that these genes confer any common structural property which promotes formation of a PL binding site. The number of antibodies in Group B is small, and none of these genes is used to encode more than one of these antibodies. It is therefore not possible to conclude that these results represent preferential use of these particular genes to encode a PL binding site in specific IgM aPL.

1.9.5 What is the extent of somatic hypermutation?

Overall, IgG aPL carried more somatic mutations than IgM aPL, and this difference was most pronounced when considering the heavy chains. All 13 published IgG V_H sequences but only five of 12 published IgG V_L sequences showed homology of 98% or less to the corresponding germline gene (see Table 1.7). The high degree of somatic mutation in IgG antibodies was expected, since class switching and somatic hypermutation are closely linked processes in the B lymphocyte. They appear to occur at similar times in B cell development within germinal centres and some important effector molecules may play major roles in both processes (Muramatsu et al., 2000).

Nevertheless, somatic hypermutation can take place in the absence of class switching, leading to the production of IgM antibodies carrying many mutations. A number of such antibodies were seen in Group A, where seven of 15 V_H sequences and two of eight published V_L sequences had homology values less than or equal to 98%.

The more specific IgM aPL in Group B, however, contained very few somatic mutations with one V_H chain having 96% nucleotide homology and the other six V_H chains having 100% homology. Five of the seven V_L chains showed greater than 99% homology to germline genes with only one V_L chain having less than 98% nucleotide homology.

1.9.6 Are the somatic mutations antigen driven?

Results of statistical analysis of the distribution of R and S mutations in the CDR and FR regions of the V_H and V_L sequences by the multinomial (Pm) method (Lossos et al., 2000) are shown in Table 1.8. A Pm value of less than 0.05 for either FRs or CDRs in the heavy or light chain is taken as representing strong evidence of antigen drive. Eight of the 14 IgG aPL analysed fulfil this criterion.

The six IgG aPL which do not have Pm values of less than 0.05 in either chain are LJ-1, AH-2, DA-3, 519, RR-7F and P11. No conclusion can be made as to whether antigen drive plays a role in the development of LJ-1 and RR-7F, because the sequences of LJ-1 V_H and RR-7F V_L are unpublished. There is good evidence that AH-2 and DA-3 are derived from a clone of B cells which has been subject to antigen driven expansion, despite the non-significant Pm values. AH-2 and DA-3 are distinct monoclonal antibodies derived from descendants of the same B lymphocyte clone in a single patient. This is evident because they share exactly the same V_H and V_L sequences, except at a few sites of somatic mutation. The fact that the same clone has given rise to two distinct monoclonal antibodies implies that it is a highly expanded clone, and the fact that the sequences contain multiple somatic mutations implies that this has been driven by antigen. Thus, in only two IgG antibodies (P11 and 519) was the evidence clearly against the influence of antigen drive.

It is therefore apparent that most of the IgG aPL have been subject to antigen driven expansion and accumulation of somatic mutations. Since these antibodies showed specificity for either PL or β_2GPI, these seem likely to have been the driving antigens.

Antibody		Heavy chain	Light chain
		Pm	Pm
C6B2	FR	0.0186	NP
	CDR	0.882	NP
C119	FR	0.00031	ND
	CDR	0.0271	ND
B122	FR	0.137	ND
	CDR	0.899	ND
B6204	FR	0.0142	ND
	CDR	0.0645	ND
BH1	FR	ND	0.560
	CDR	ND	0.908
RSP4	FR	0.422	ND
	CDR	0.942	ND
R149	FR	0.00009	ND
	CDR	0.00177	ND
AH-2	FR	0.435	ND
	CDR	0.152	ND
DA-3	FR	0.349	ND
	CDR	0.122	ND
UK-4	FR	0.150	0.035
	CDR	0.622	0.016
516	FR	0.0376	0.101
	CDR	0.286	0.433
519	FR	0.982	ND
	CDR	0.844	ND
B9416	FR	0.0029	ND
	CDR	0.484	ND
HL-5B	FR	$<1x10^{-6}$	NP
	CDR	0.0213	NP
RR-7F	FR	0.0739	NP
	CDR	0.0645	NP
B14	FR	0.317	0.041
	CDR	0.104	0.873
B22	FR	0.011	ND
	CDR	0.0911	ND
B27	FR	0.011	$<1x10^{-6}$
	CDR	0.0911	0.0367
P11	FR	0.0820	0.175
	CDR	0.641	0.229

Table 1.8 Statistical analysis of aPL with < 98% nucleotide homology to germline genes

For origin, sequence features and binding characteristics of each mAb see Tables 1.6 and 1.7.

It must be stressed however, that all statistical models for determining the influence of antigen drive have drawbacks. For example, they do not take into account the fact that some FR residues contact the antigen while some CDR residues do not. Antigen drive might select for mutations at such FR residues and against mutation at such CDR residues. The statistical model would interpret such selection as evidence against antigen drive. In addition, the specification of $p=0.05$ as the threshold for significance is somewhat arbitrary in this context.

The results for the more specific IgM aPL in Group B are completely different from those of the IgG aPL. Most have very few mutations, and even the two sequences which are significantly mutated (RSP-4 V_H and BH1 V_L) do not show any evidence of antigen drive on statistical analysis and did not demonstrate specificity for PL or β_2GPI. Indeed, the majority of the more specific IgM aPL had very few somatic mutations. This suggests that these IgM attain specificity due to features inherent in their germline sequence or produced at the V_H-D_H, D_H-J_H or V_L-J_L junctions. Antigen drive would then tend to select against somatic mutations because mutations would tend to alter the favourable germline sequence. Conversely, IgG aPL would attain specificity and high affinity for PL or β_2GPI due to accumulation of somatic mutations.

Seven of the polyreactive IgM aPL could potentially have been subjected to statistical analysis, since either their V_H or V_L sequences showed less than 98% homology to germline genes. However, for three of these antibodies (H3, H5 and A5) nucleotide sequences were not published and therefore could not be analysed (Hohmann et al., 1995). Of the remaining four antibodies (C6B2, C119, B122 and B6204), statistical analysis gave Pm values of less than 0.05 in three cases. This shows that antigen driven accumulation of mutations in IgM antibodies is not always associated with the loss of polyreactivity. However, extensive somatic mutation was the exception rather than the rule in polyreactive IgM antibodies, in contrast to the IgG aPL discussed above.

1.9.7 Are particular residues important in CDRs and contact regions?

The sequences of the V_H regions of the more specific IgM and IgG aPL are shown in Figure 1.3 whilst the sequences of the V_L regions of these aPL are shown in Figure 1.4.

```
     FR1                                 CDR1      FR2              CDR2
                                          ab                          abc
IgM  1        10          20         30  31    35  36           47  5052          58     65
BH1  QVQLVESGGGVVQPGGSLRLSCAASGFTFS  S--YGMH  WVRQAPGKGLEWVA  FIRY--DGSNKYYADSVKG
RSP4 QVQLVESGGGVVQPGRSLRLSCAASGFTFS  S--YGMH  WVRQAPGKGLEWAA  VISY--DGSNKYYADSVKG
REN  QVQLQESGPGLVKPSQTLSLTCTVSGGSIS  SGSYSWS  WIRQPAGKGLEWIG  RIY---TSGSTNYNPSLKS
Kim  QVQLVQSGAEVKKPGSSVKVSCKASGGTFS  S--YAIS  WVRQAPGQGLEWMG  GIIP--IFGTANYAQKFQG
STO  QVQLQESGPGLVKPSETLSLTCTVSGGSVS  SGSYYWS  WIRQPPGKGLEWIG  YIY---YSGSTNYNPSLKS
9421 EVQLVESGGGLVQPGGSLRLSCAASGFTFS  D--HYMD  WVRQAPGKGLEWVG  RTRNKANSYTTEYAASVKG
9427 EVQLVESGGGLVKPGGSLRLSCAASGFTFS  N--AWMS  WVRQAPGKGLEWVG  RIKSKTDGGTTDYAAPVKG
IgG
516  QVQLVQSGAEVKKPGSSVKVSCKTSGGTFS  S--YAIN  WVRQAPGQGLEWMG  GIIP--IYGTANYAQKFQG
519  QVQLQQWGAGLLKPSETLSLTCAVYGGSFR  G--YYWS  WIRQPPGKGLEWIG  EIN---HSGSTNYNPSLKS
R149 QVQLVQSGAEVKKPGSSVKVSCKASGGTFS  S--YSVS  WVRQAPGQGLEWMG  RIIP--VLTLANYAQKFQG
B22       LESGPGLLKPSQTLSLTCVISGDNVS  RNSVGWN  WLRQSPSRGLEWLG  RTYYR-SKWYNDYAAFVRS
P11       LESGPGLVKPSQTLSLTCTVSGDSIS  SGGYYWS  WVRQHPGKGLDWIG  YIY---YSGKRYYNPSLKS
B14       LESGEGVVQPGRSLRLSCAASGFTFR  S--YAMH  WFRQTPGRGLEWVS  GISG--TGGSTDYANSVRG
RR7F          GGLVQPGGSLRLSCAASGFSFS  S--YVMS  WVRQAPGKGLEWVS  TISG--SGASTYYADSVKG
HL5B          GDLIQPGGSLRLSCAASGFTVT  D--THMN  WVRQAPGKGLEWVS  VIY---KGGRSDYADSVKG
9416 EVQLLESGGGLVQPGGSLRLSCAASGFTFS  S--YVMS  WVRQAPGKGLEWVS  AISG--SGGSTYYADSVKG
AH2  EVQLLQSGAEVKKPGESLKISCQASGYSFA  T--YWIG  WVRQMPGKGLECMG  MVFP--GDSDTRYSPSFQG
DA3  EVQLLQSGAEVKKPGESLKISCQASGYSFA  T--YWIG  WVRQMPGKGLECMG  MVFP--GDSDTRYSPSFQG
UK4  EVQLVESGGGLVQPGGSLRLSCVASGFTFS  G--YWMH  WVRQVPGKGLVWVS  RINS--DGRSTSYADSVKG

                              CONTACT H1                  CONTACT H2


     FR3                                 CDR3                         JH
                    abc                        abcdefghijk 102
IgM  66              82         90 93     95    100
BH1  RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAN    NNGQQLVYS------YMDV       WGKGTTVTVSS
RSP4 RFTISRDNAKNSLSLQMNSLRAEDTAMYYCAR    NRWEPLN------------       WGQGTLVTVSS
REN  RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR    DKGGFWSGYYTRNSRAAFDI      WGQGTMVTVSS
Kim  RVTITADKSTSTAYMELSSLRSEDTAVYYCAR    ARIAARLPGSN----WFDP       WGQGTLVTVSS
STO  RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR    VGTRGLWLFY--------Y       WGQGTLVTVSS
9421 RFTISRDDSKNSLYLQMNSLKTEDTAVYYCAR    VPPYYYDSSGYYPD AFDI       WGQGTMVTVSS
9427 RFTISRDDSKNTLYLQMNSLKTEDTAVYYCTT    DYSGYYPW-------YFDY       WGQGTLVTVSS
IgG
516  RVTITADESTSTAYMELSSLRSGDTAVYYCAR    GGRFIRGLT------FMDV       WGQGTTVTVSS
519  RVTISVDTSKNQFSLNLRSVTAADTAIYFCAR    GRIAVTSQPRP-----FDS       WGQGILVTVSS
R149 RVTITADKSTSTAYMELSSLRSEDTAVYYCAR    GSIRRERFLDRIQTRESEGSYYAMDVWGQGTTVTVSS
B22  RITINPDTSKNQFSLHLNSVTPEDTAVYYCAR    GAGAAPH----------DY       WGQGTLVTVSS
P11  RVTILLDLSKNQFSLKLNSVTAADTAVYYCAR    GPYALFGVVIPH---YFDN       WGQGALVTVSS
B14  RFTISRDNSKDTLYLQMNNLRVEDTAVYYCSK    QVFF                      GGQGALVTVSS
RR7F RFTISRDNSKNTLYLQMNSLRADDTAVYYCAK
HL5B RFTISRENSRNTLYLQMNSLRVDDTAVYYCAR
9416 RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK    EAPAGGGEY                 WGQGTLVTVSS
AH2  QVTISADKSLSTAYLQWSSLKASDTAIYLCAR    LGTGAPLTAFFTP      YYYSRMDVWGQGTTVTVSS
DA3  QVTISADKSLSTAYLQWSSLKASDTAIYFCAR    LGTGEPLTANFTPY      YYSRMEVWGQGTTVTVSS
UK4  RFTISRDNSKNTLVLQMNSLRADDTAVYYCAR    DLGAVTAY-------YFDY       WGQGALVTVSS

                                           CONTACT H3
```

Figure 1.3 Sequence alignment of more specific IgM and all IgG aPL heavy chains

The positions of CDRs and FRs are as defined by Wu and Kabat (1970). Positions of the contact regions H1, H2, H3, L1, L2 and L3 are as defined by MacCallum *et al.* (1996). The underlined amino acids are somatically mutated and those shown in bold are at sites that contact antigens.

KAPPA

```
     FR1                        CDR1              FR2              CDR2     FR3                                CDR3       Jκ
                                      abcdef                                                                        ab
IgM  1        9           20    24    30          34            46    50     56   60          70         80          89      95 96
Kim  EIVLTQSPATLSLSPGERATLSC   RASQSVS------SYLA WYQQKPGQAPRLLIY DASNRAT GIPARFSGSGSGTDFTLTISSLEPEDFAVYYC QQRSNWPL-T FGGGTKVEIK
9427 DVVMTQSPLSLPVTLGQPASISC   RSSQSLVYSDGN-TYLN WFQQRPGQSPRRLIY KVSNRDS GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC MQGTHWPF-T FGPGTKVDIK
STO  DIVMTQSPDSLAVSLGERATINC   KSSQSVLYSSNNKNYLA WYQQKPGQPPKLLIY WASTRES GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC QQYYSTPPGT FGQGTKLEIK
IgG
516  DIQMTQSPSSVSASVGDRVTITC   RASQGIS------NWLA WYQQKPGKAPKLLIY AASSLQS GVPLRFSGSGSGTDFTLTISNLQPEDSATYYC QQAKSFPPVT FGGGTKVEIK
519  EIVMTQSPATLSVSPGERATLSC   RASQSVS------SNLA WYQQKPGQAPRLLIY GASTRAT GIPARFTGSGSGTEFTLTISSLQSEDFAVYYC QQYNKWPL-A FGGGTKVEIK
B27  ELQMTQSPPSLSASVGDRVTITC   RASQGIS------TYLV WYQQKPEKAPKSLIY AASNLQS GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYNSYPF-T FGGGTKVEIK
B14  ELQMTQSPSSLSASVGDRVTITC   RASQGIS------NYLA WYQQKPGKVPKLLIY AASTLQS GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQSYSTPR-T FGQGTKVDIK
P11  ELHMTQSPSSLSASVGDRVTITC   RASQGIS------SALA WYQQKPGKAPKFLMY AASTLQS GVPSRFSGSGSGTEFTLTISSLQPEDFGTYYC QQFNAYPL-T FGPGTKVEIK
B22  ELQMTQSPSSLSASVGDRVTITC   RASQGIS------NYLA WYQQKPGKVPNLLIY GASTLQS GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC QKYDSAPR-T FGPGTKVDIK
9416 DIQMTQCPSSLSASVRDRVTITC   RASQSIS------SYLN WYQQKPGKAPKLLIY AASSLQS GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQSYSTPPWT FGQGTKVEIK
R149 DIVMTQSPLSLPVTPGEPASISC   RSSQSLLHRYGY-NYLD WYLQKPGQSPQLLIY LGSNRAS GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC MQALQTPY-T FGQGTKLEIK

                                  CONTACT L1          CONTACT L2                                        CONTACT L3
```

LAMBDA

```
     FR1                       CDR1            FR2              CDR2     FR3                                CDR3              Jλ
                                     Abc                                                                            abcdef
IgM  1        9          20   24    30        34            46     50     56   60         70        80          89      95   96
BH1  FYELTQPPSVSVSPGQTASITC SG-DK-LGDK-YAC WYQQKPGQSPILVIY QDSKRPS GIPERFSGSNSGNTATLTISGTQAIDEADYYC QAWDSSTA---V FGTGTKVTVLG
REN  QTVVTQEPSFSVSPGGTVTLTC GLSSGSVSTSYSPS WYQQTPGQAPRTLIY STNTRSS GVPDRFSGSILGNKAALTITGAQADDESDYYC VLYMGSGIW--V FGGGTKLTVLG
9421 QSALTQPASVSGSPGQSITISC TGTSSDVGGYNYVS WYQQHPGKAPKLMIY DVSNRPS GVSNRFSGSKSGNTASLTISGLQAEDEADYYC SSYTSSST---R FGGGTKLTVLG
RSP4 QSVLTQPPSVSGAPGQRVTISC TGSSSNIGAGYDVH WYQQLPGTAPKLLIY DNNNRPS GVPDRFSGSKSGTSGSLAITGLQAEDEADYYC QSYDSSLSG--V FGTGTKVSVLG
IgG
LJ1  SYELTQPPSVSVAPGKTARITC GG-NN-IGSK-NVH WYQQKPGQAPVLVIY DDSDRPS GIPERFSGSNSGNTATLTISRVEAGDEADYYC QVWDSSSDQ---  FGGGTKLTVLG
AH2  QSVLTQPPSVSGAPGQRVTISC TGSSSNIGAGYDVH WYQQVPGTAPKLLIY GKSNRPS GVPDRFSGSKSGTSASLAITGLQAEDEADYYC QSYDSSLSG-WV FGGGTKLTVLG
DA3  QSVLTQPPSVSGAPGQRVTISC TGSSSNIGAGYDVH WYQQVPGTAPKVLIY GKSNRPS GVPDRFSGSKSGTSASLAITGLQAEDEADYYC QSYDSSLSG-WV FGGGTKLTVLG
UK4   SALTQPASVSGSPGQSITISC TGTSNDVGSYNYVS WYQLHPGEAPKLLIY DAIKRPS GVSNRFSGSESGNTASLTISGLQAEDEGDYYC SSYTNR----VF FGGGTKLTVLG

                                CONTACT L1          CONTACT L2                                       CONTACT L3
```

Figure 1.4 Sequence alignment of IgM and IgG aPL light chains

See legend to Figure 1.3.

The figures show the positions of the CDRs and FRs identified by the Kabat system (Wu and Kabat, 1970) and contact sites as defined by MacCallum *et al.* (1996). Most of these contact sites are within the CDRs but it is important to note that not all CDR residues are contact sites and that some FR residues can be contact sites.

On considering the figures, it is apparent that somatic mutations occur frequently in the contact H1 and H2 regions of the IgG antibodies, less commonly in L1, L2 and L3 of the IgG antibodies, and are very rare in the IgM antibodies. None of the residues in H3 have been assigned as somatic mutations since it is very difficult to be sure where somatic mutations occur in H3, due to the contribution of junctional diversity and the large number of cases in which germline D genes of origin cannot be identified.

The hypothesis that somatic mutation to create Arg, Lys and Asn residues in the contact regions may play a major role in IgG aPL is examined in Table 1.9. The proportion of somatic mutations in the contact regions which give rise to these amino acids is compared with the proportion of mutations in the same regions which give rise to aspartic acid (Asp) or glutamic acid (Glu). These residues were chosen for comparison because thay are acidic and negatively charged as opposed to the basic, positively charged Arg and Lys.

Table 1.9 clearly shows an excess of mutation to Arg, Lys and Asn in the contact sites of the IgG aPL in comparison to Asp and Glu. This is particularly striking in V_HCDR1 and V_HCDR2. In Table 1.10, the total numbers of Arg, Asn and Lys residues in the contact regions of both the IgG aPL and the more specific IgM aPL are compared with the numbers of Asp and Glu residues in those regions. This Table includes both germline encoded residues and those derived by somatic mutation. This comparison is particularly important for the IgM antibodies, where somatic mutation plays no major role. In general, there is an excess of Arg, Lys and Asn residues over Asp and Glu residues in the contact regions of the V_H and V_κ, but not the V_λ sequences. Interestingly, this does not hold true for the H3 regions of the IgG antibodies. Accumulation of basic residues (particularly Arg) in H3 has been noted to be a common feature of human and murine IgG anti-DNA antibodies (Radic and Weigert, 1994; Rahman et al., 1998), but does not seem to be an important feature in the IgG aPL considered here.

V Region	Number of antibodies	Number of somatically mutated contact residues that are Arg, Asn or Lys / number of contact residues that are somatic mutations			Number of somatically mutated contact residues that are Asp or Glu / number of contact residues that are somatic mutations		
		Contact Region 1	Contact Region 2	Contact Region 3	Contact Region 1	Contact Region 2	Contact Region 3
IgG V_H	12	5 / 20	4 / 22	-	1 / 20	2 / 22	-
IgG V_κ	8	2 / 7	1 / 3	2 / 5	0 / 7	0 / 3	1 / 5
IgGV_λ	4	1 / 1	1 / 5	2 / 2	0 / 1	2 / 5	0 / 2

Table 1.9 Analysis of somatic mutations at contact residues

V Region	Number of antibodies	Total Arg, Asn or Lys residues present at contact sites (in brackets; total not including somatically mutated residues)			Total Asp or Glu residues present at contact sites (in brackets; total not including somatically mutated residues)		
		Contact Region 1	Contact Region 2	Contact Region 3	Contact Region 1	Contact Region 2	Contact Region 3
IgG V_H	12	6 (1)	15 (11)	9 (-)	1 (0)	9 (7)	12 (-)
IgG V_κ	8	7 (5)	2 (1)	5 (3)	1 (1)	0 (0)	1 (0)
IgGV_λ	4	3 (2)	3 (2)	2 (0)	2 (2)	2 (1)	2 (2)
IgM V_H	7	1 (1)	13 (13)	11 (-)	2 (2)	5 (5)	8 (-)
IgM V_κ	3	5 (5)	4 (4)	2 (2)	1 (1)	3 (3)	0 (0)
IgM V_λ	4	2 (2)	5 (4)	1 (1)	2 (2)	2 (0)	2 (2)

Table 1.10 Analysis of all amino acids present at sites of contact

The number of Arg, Lys and Asn is compared to the number of Asp and Glu.

Thus, whereas both the Group B IgM aPL and the IgG aPL show some evidence of accumulation of Asn, Arg and Lys at the contact sites, these are mostly encoded in the germline in IgM and derived from somatic mutation in IgG. The presence of CDRs and contact regions which are rich in Arg, Asn and Lys residues in both the more specific IgM aPL and IgG aPL is relevant to the nature of the epitope bound by these antibodies. It has already been discussed how pathogenic aPL have a predilection for negatively charged PL and β_2GPI (section 1.2.5) which has a potential epitope rich in negatively charged residues within domain I (section 1.6.6). Therefore aPL containing Arg, Asn, and Lys residues in the contact regions may increase the affinity of binding via electrostatic interactions and hydrogen bonds with negatively charged epitopes upon PL and domain I of β_2GPI.

1.9.8 Are there differences between antibodies which bind β_2GPI and those which do not?

Eight antibodies were either known to bind β_2GPI or there was strong evidence to suggest that β_2GPI was required for them to bind phospholipids. Three of these antibodies (BH1, REN and B9427) were IgM antibodies from group B while the other 5 (516, B14, B22, B27 and P11) were IgG antibodies. When these antibodies are considered as one group, the results are similar to those noted for the whole set of aPL. Thus the five IgG antibodies which bound β_2GPI all had many somatic mutations and Pm values suggested antigen drive in four cases (P11 was the exception). The three IgM antibodies had few or no mutations with no evidence of antigen drive. All the IgG antibodies except P11 contained somatic mutations to Arg, Asn or Lys in their contact regions whereas none of the IgM antibodies had mutations to those residues in their contact regions.

Since this updated aPL sequence analysis was completed, the sequences of seven further IgG aPL (four of which were β_2GPI dependent) have been published (Chukwuocha et al., 2002). Five of the antibodies had previously been shown to be thrombogenic whilst two were not, in a mouse model (Pierangeli et al., 2000). The pattern of gene usage in these aPL did not reveal any bias, consistent with the above findings. Thirteen out of the 14 Ab heavy/light chains contained somatic mutations with 84 -97% homology. In six of the seven aPL statistical analysis using the multinomial method (Lossos et al., 2000) showed evidence of antigen drive in the FRs and/or CDRs. The presence of antigen driven somatic mutations was found in both pathogenic and non-pathogenic aPL. An accumulation of Arg residues however, was found in the V_H CDR3 of pathogenic compared to non-pathogenic aPL (Chukwuocha et al., 2002). Thus the findings from sequence analysis of these additional aPL was consistent with those described above.

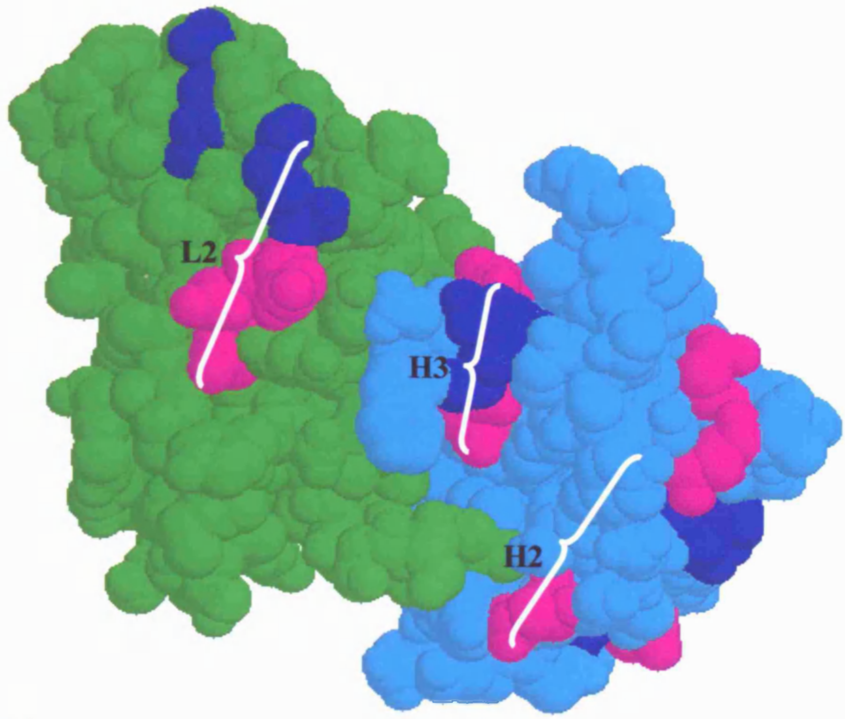
1.9.9 Computer modelling of aPL

Computer models of the specific IgM aPL RSP4 and the IgG aPL UK-4 are shown in Figure 1.5. These models support the idea that Arg and Asn residues are important at the binding sites of these antibodies. In RSP4, Arg and Asn residues from a number of different CDRs form ridges on the surface of the molecule. In UK-4, Arg and Asn residues from L1, H2 and L3 are exposed at the surface. Two of these Asn residues are derived from somatic mutations in the light chain.

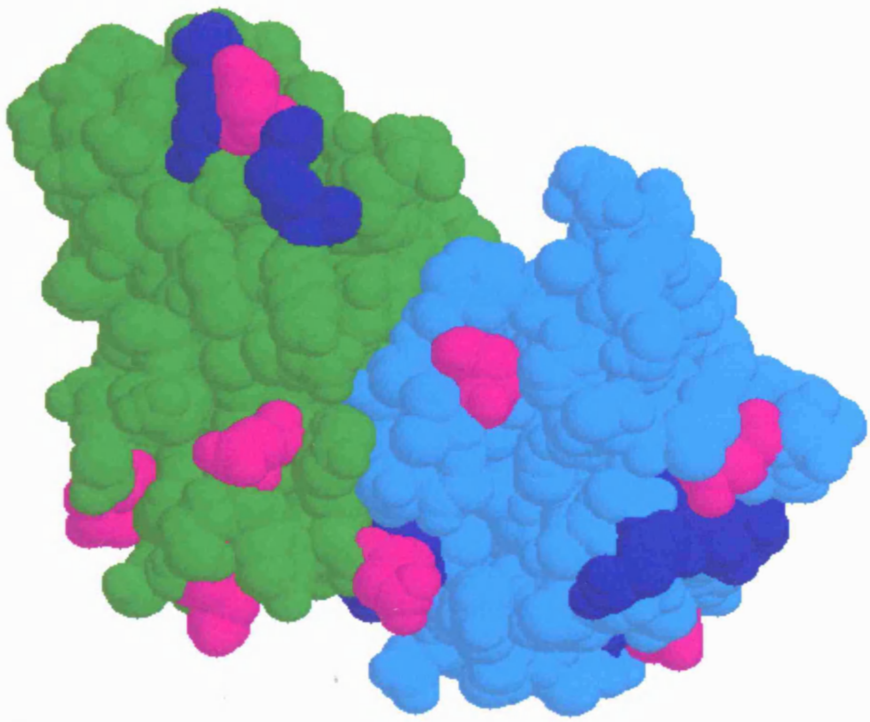
Once computer models such as these have been used to identify particular amino acids that potentially affect the aPL- β_2GPI-PL interaction, further experiments are needed to produce variant forms of aPL in which these key residues are altered to test the effect this has upon binding and/or pathogenic properties of the antibody. Of course this is only possible if the wild type and variant antibodies can be expressed as whole antibody molecules and their properties compared. A number of different systems to express antibody sequences exist.

1.10 EXPRESSION SYSTEMS FOR ANTIBODIES

Compared to monoclonal Ab produced from PBL or splenic lymphocytes from humans or mice the *in-vitro* expression of cloned Ab cDNA produces a poor yield of Ig. A powerful advantage of expression systems, however, is that the sequence of the DNA being expressed can be controlled. It is possible to determine the importance of particular residues in antigen binding by expressing cDNA encoding the H and L chains of a particular Ab *in-vitro* and then using a technique such as site-directed mutagenesis to create altered forms of the sequence in which Arg, Asn or Lys residues of interest had been altered. For instance, if an aPL were altered in this way the binding properties of these altered forms could then be tested to see whether affinity for PL and/or β_2GPI had changed in the way predicted by the hypothesis. Similar experiments have shown the importance of Arg residues in human and



A) RSP4



B) UK4

Figure 1.5 Computer generated models of the three dimensional structures of RSP4 and UK4
The three dimensional orientation of UK4 is exactly like that of RSP4 so location of the contact regions can be directly cross-compared.
Colour code:- Green – V_L; Light Blue – V_H; Magenta – Asn residues; Purple – Arg residues

murine anti-DNA antibodies (Radic et al., 1993; Radic and Weigert, 1994; Rahman et al., 2001).

All expression systems involve cloning V_H and/or V_L sequences of a selected antibody into an expression vector containing the appropriate amount of C_H and/or C_L as well as the components required to enable the plasmid vector to express Ig protein once transfected into either a bacterial or eukaryotic cell. In this way, either part or the whole Ig molecule is expressed containing a functional binding site. Several different types of expression system have been used for this purpose and they will be described below.

1.10.1 Bacterial expression systems

A major disadvantage of bacterial expression of eukaryotic proteins is that post translational modifications such as glycosylation are absent and that the molecules may not fold properly. Consequently it has not been possible to express a stable, functional whole Ig molecule in bacteria (Rahman et al., 2002b). Since binding properties of an Ab depend only on the V regions however, it has proved possible to express smaller fragments of Ab which contain a functional binding site.

The simplest expression products are single chain fused variable region (scFv) molecules. In scFv expression plasmids V_H and V_L sequences are cloned either side of a sequence encoding a short flexible linker fragment, see Figure 1.6 A). When expressed from bacterial cells the flexibility of the linker, typically rich in glycine and serine residues, allows the V_H and V_L to interact with each other and produce an antigen binding site (Bird et al., 1988).

In some scFv expression systems the V_H and V_L DNA are ligated into phagemids rather than plasmid vectors. This system enables scFv to be expressed as a fusion protein connected covalently to a surface protein of the phage particle and is called phage display. If a library of V_H and V_L sequences is cloned into a phagemid a library of different phage particles that display a repertoire of different V_H/V_L combinations is produced. This process is called repertoire cloning (McCafferty et al., 1990). By panning phages against surfaces carrying antigens of interest (e.g DNA or PL) under gradually more stringent washing conditions in each round of exposure to antigen it is possible to select from a large repertoire of combinations those with a high affinity for a particular antigen. A drawback of

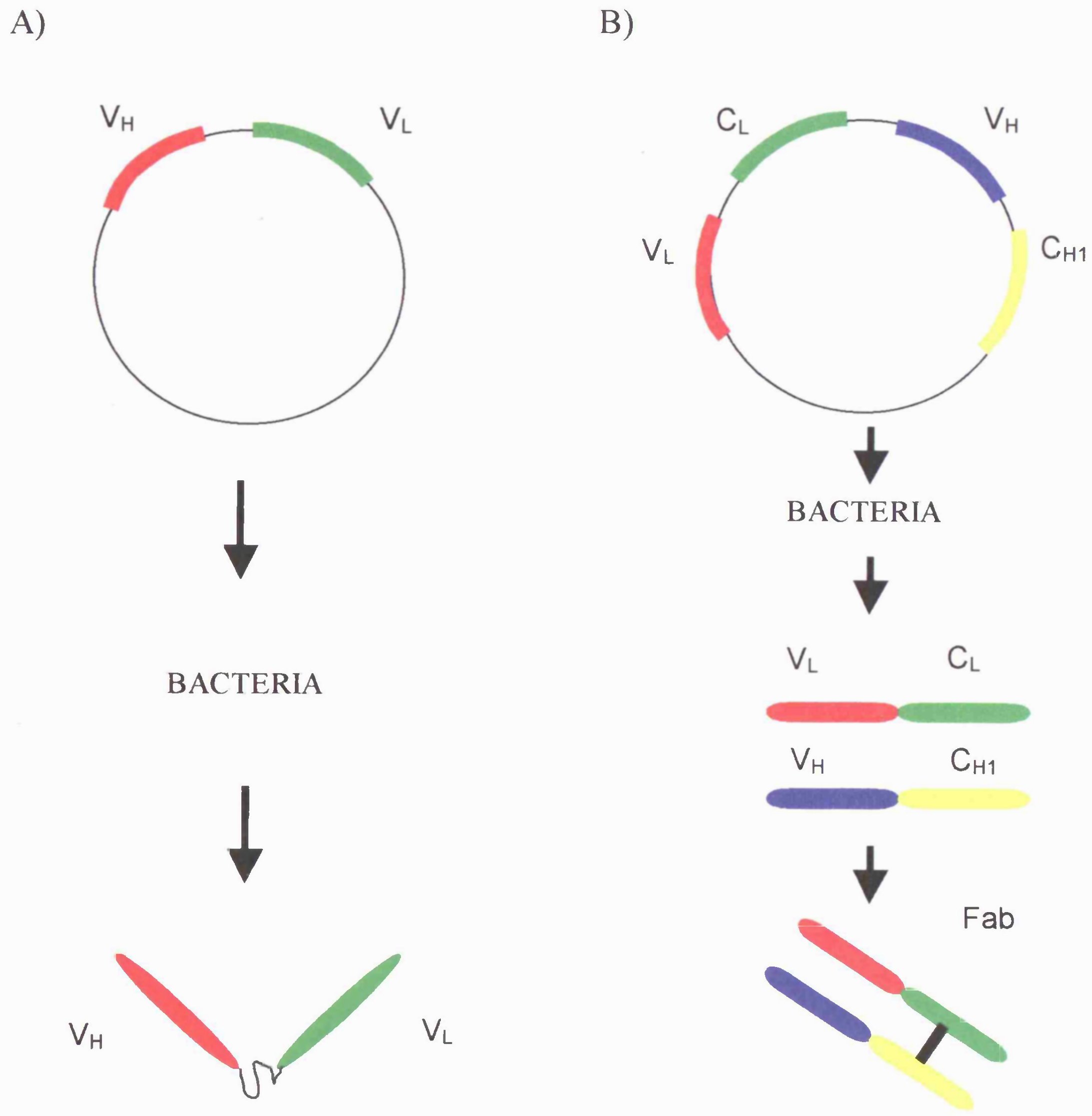


Figure 1.6 Overview of bacterial expression of antibody fragments

Schematic representation of the use of bacterial expression systems to produce: - A) single chain Fv molecules (scFv); and B) Fab.

this technique however is that the V_H /V_L gene combinations created are artificial and not all may have been expressed in patient serum. This drawback does not apply to monoclonal aPL that are derived from immortalised B cells in which the V_H /V_L gene combination is definitely expressed by at least one B cell in the individual from which it was produced.

An alternative to scFv is to express Fab fragments from bacteria, either in soluble form or on the surface of a phage particle, as described above. Expression vectors for these fragments contain V_H and V_L followed 3` by a C_H1 sequence and a C_L sequence respectively, see Figure 1.6 B). Hence the products of expression are the whole light chain and half the heavy chain which combine covalently to form Fab (Better et al., 1988). Fab are the most appropriate antibody fragments for crystallization studies, particularly if they can be produced and purified from large scale bacterial cultures.

Fab and scFv molecules however, are both unsuitable for use in functional assays because they lack some or all C region domains. Therefore despite the usefulness of these antibody fragments in determining the binding properties of the V region of an antibody they cannot be used to investigate how altering these binding properties may affect pathogenicity of these antibodies *in-vivo*. Whole IgG molecules are required for this purpose. Thus it is necessary to transfect cultured eukaryotic cells to express whole IgG (Rahman et al., 2002b).

1.10.2 Eukaryotic expression systems

The expression of whole Ig in mammalian cells may be achieved in a variety of ways which give rise to either transient expression of antibody or the selection of stably transfected cell lines. In transient expression systems the expression vectors are not preserved in host cells for more than a few days, so the total yield of antibody is generally low. This form of expression allows rapid screening of large combinations of different Ab and does not require the selection of cells for drug resistance markers or long term maintenance of cell lines. In contrast, in stable expression systems transfected expression plasmids will be incorporated into the genome of a small proportion of the host cells, selected using markers such as drug resistance, and be maintained there over many generations. This system allows the production of much larger quantities of Ab than with transient expression but is more difficult and time consuming to achieve, hence fewer Ab may be expressed.

To produce whole antibody, expression vectors are created which encode for entire H and L chains. The H chain expression vectors contain V_H sequence DNA 5` to the appropriate $C_H$ DNA sequence, thus encoding the entire H chain constant region and hinge region. Similarly, in L chain expression vectors $V_L$ is cloned 5` to C_L. The H and L vectors can then be co-transfected into eukaryotic cells and whole fully glycosylated IgG molecules are expressed, see Figure 1.7 A). This method makes it easy to carry out chain shuffling experiments in which the same H (or L) chain is paired with a range of different partner chains. Alternatively, the H and L chain constructs can be combined into a single vector, see Figure 1.7 B). An advantage with this method is that a cell does not have to acquire two separate plasmids simultaneously and an increased yield of Ig may be obtained although chain shuffling is not so readily performed. Lastly, a vector containing only H chain may be transfected into hybridoma cells that secrete L chain only, called H chain loss variants (HCLV), see Figure 1.7 C).

1.10.3 The use of different expression systems in the further investigation of links between sequence, structure and function of anti-DNA antibodies

Therefore the most suitable expression system for analysis of an Ab depends upon the precise hypothesis being tested. If the effects of altering the Ab binding site upon its ability to bind antigen is being examined, then bacterial expression of antibody fragments as either scFv or Fab is appropriate. In particular, the expression of either of these fragments by phage display allows large scale chain shuffling experiments to take place investigating the relative contributions of the V_H and V_L sequences to antigen binding. Alternatively, if it is supposed that an alteration in expressed Ab sequence will affect the functional properties of that Ab, then a eukaryotic system must be used to produce large quantities of whole Ig for use in an appropriate biological assay.

Various different expression systems have been used to study the relationship between sequence, structure and function of murine and human anti-dsDNA Ab. It is instructive to consider the results of some of these experiments before considering their suitability for the study of aPL.

Bacterial expression systems have been used to produce high affinity anti-DNA Ab by repertoire cloning. To investigate whether the random H/L chain combinations in the Fab

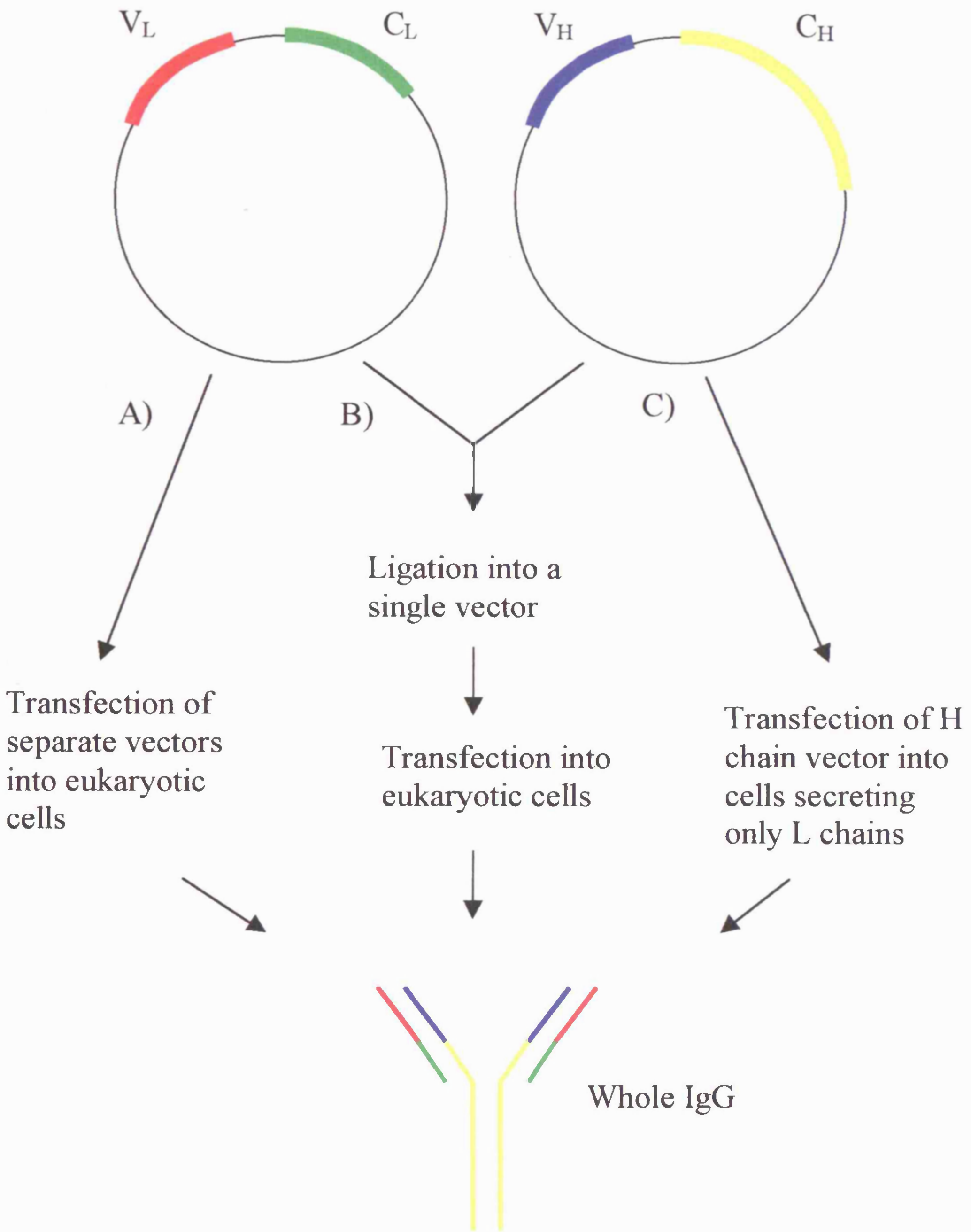


Figure 1.7 Expression of whole IgG in eukaryotic cells

Schematic representation of the production of whole IgG molecules in eukaryotic cells by transfection of: - A) separate heavy and light chain expression vectors; B) a single vector containing both heavy and light chain sequences; and C) a heavy chain expression vector into cells which secrete only light chains.

produced represented combinations found *in-vivo,* one group (Roben et al., 1996) have used anti-idiotype antibodies. Idiotypes are antigenic determinants found in the V region of antibodies that can be recognised by specific antisera. Few anti-idiotypes identify the gene segment used to encode the antibody chain; however antibodies with the same particular idiotype should in theory have the same amino acid sequence at the part of the sequence that encodes the idiotype. Therefore two antibodies which have the same H chain idiotype and the same L chain idiotype are likely to have very similar H and L chain sequences.

Roben *et al.* (1996) obtained IgG anti-dsDNA Fab from the PBL of two identical twin sisters, one of whom had clinically active SLE, by repertoire cloning. One of these Fab, AD4-37 derived from the sister with SLE, carried the H chain idiotype B6 and the L chain idiotype λIIIa. Using anti-idiotype antibodies in an inhibition ELISA to test the serum of the patient from whom AD4-37 was derived, it was found that both of these idiotypes did occur on the same circulating anti-DNA antibody in this patient.

In the same experiment, another anti-dsDNA Fab, AD4-18, was derived from PBL of the sister with SLE. Both of these Fab, AD4-37 and AD4-18, had high affinity for dsDNA and contained accumulations of basic residues in their V_HCDR3. The sequence of AD4-37 was near germline in both its V_H and V_L regions, whereas AD4-18 contained multiple mutations in both. To determine the contributions of the AD4-37 V_H and V_L sequences to DNA binding affinity, the library of L chain sequences from each of the twins was ligated into an expression plasmid containing the sequence of AD4-37V_H. This process enabled the production of a library of phages carrying AD4-37V_H with many different L chains. Analagous experiments were carried out to produce libraries in which AD4-37V_L was paired with many different H chains. AD4-37V_H was able to form anti-dsDNA Fab only in combination with some V_λ (but not V_κ) sequences from either sister. Whilst AD4-37V_L only formed anti-dsDNA Fab with V_H sequences from the twin with SLE, and almost all of these contained CDR3 sequences identical to that of AD4-37V_H. These results suggested that the presence of the V_H sequence (particularly CDR3) was the dominant factor in determining the ability of AD4-37 to bind DNA.

HCLV have been used to study the importance of Arg residues in murine anti-DNA Ab 3H9 (Radic *et al.*, 1991; Radic *et al.*, 1993). The H chain of 3H9 contains three somatic mutations in CDR2, one of which creates an Arg residue. It also contains an Arg in CDR3, which results from N-addition at the V_HD_H junction. Transfection of an expression vector containing the sequence of $3H9V_H$ into a range of different HCLV showed that it could form an anti-DNA binding site in association with several different L chains (Radic et al., 1991). Mutagenesis of the sequence of $3H9V_H$ in the expression vector to remove the Arg in CDR2 and CDR3 led to a large reduction in affinity for DNA, whereas reversion of the other somatic mutations in CDR2 did not do this (Radic et al., 1993). Furthermore, the introduction of extra Arg into $3H9V_H$ at sites where Arg were known to occur in the CDRs of other murine anti-DNA Ab led to an increase in affinity for DNA by up to 70-fold. The relationship between the number of Arg residues and DNA binding however was not a simple one. For example, the introduction of an extra Arg at position 64 of $3H9V_H$ eliminated binding to DNA, probably due to formation of a salt bridge with an adjacent aspartic acid residue.

Further experiments have also shown that positively charged residues (such as Arg) in the CDRs are not the only factors which influence binding to DNA. A range of Ab produced by transfection of mutagenised forms of the V_H sequence of the murine anti-DNA Ab R4A into HCLV secreting $R4AV_L$ have been studied (Katz et al., 1994). Mutations in $R4AV_H$ were designed to alter the numbers of charged residues. Although loss of an Arg in CDR3 was found to reduce binding, there were other positions in the $R4AV_H$ where changes that increased positive charge led to decreased binding to DNA, or changes that decreased positive charge enhanced DNA binding. In fact, the sequence which gave the highest binding to DNA had two fewer Arg than the original $R4AV_H$ sequence and these Arg had been lost from FR3 with no changes in the CDRs at all.

Katz *et al.* (1994) also took advantage of the fact that they had expressed altered sequences as whole Ig molecules by examining the pathogenic effects of these sequence alterations. They injected young severe combined immunodeficiency mice intraperitoneally with either R4A hybridoma cells or one of the HCLV expressing whole IgG with the altered $R4AV_H$. All mice carrying the R4A hybridoma developed proteinuria with glomerular deposition of the antibody in the kidneys. The mutant cell line producing IgG with the highest anti-dsDNA ability, described above, showed some glomerular deposition but deposited to a greater extent in the tubules, although proteinuria still occurred. Therefore the change in affinity for dsDNA may also be associated with the acquisition of a novel antigenic cross-reactivity. In conclusion, these results indicate that changes in the ability to bind dsDNA do not necessarily predict changes in the pathogenic properties of an antibody.

Overall the findings from various experiments using different expression systems support the importance of basic amino acids at the anti-dsDNA binding site, particularly Arg residues in V_HCDR3 of anti-DNA Ab. The relationship of these sequence features to pathogenicity however is not straight forward, as shown by the experiments of Katz *et al.* (1994). Given that the results of sequence analysis of pathogenic aPL are similar to those of pathogenic anti-DNA Ab and demonstrate a clustering of Arg and Lys residues in their antigen binding regions (Giles et al., 2003b), the different expression systems described above would be ideal to study the relative contribution of these sequence features to antigen binding and pathogenicity.

1.10.4 Suitability of different expression systems to study aPL

There have been few studies of the relative importance of heavy and light chain sequence motifs in the binding of aPL to PL-β_2GPI. Several groups have studied murine aPL expressed as scFv. One group found the heavy chain of a pathogenic aPL to be important in conferring β_2GPI dependent binding to PL as well as LA activity (Blank et al., 1999b). Another group studied the murine anti-DNA antibody 3H9, which has dual specificity for PL (Cocca et al., 2001). They found that the introduction of Arg residues into the heavy chain of 3H9 variable region at positions known to mediate DNA binding enhanced binding to PS-β_2GPI complexes. In contrast, the CL binding activity of different monoclonal antibodies A1.72 and A1.84 was found to be solely mediated by the light chain sequence (Pereira et al., 1998). The direct relevance of these studies to APS in humans is limited however by the use of murine antibodies in the form of scFv, which do not contain any constant region sequence, and are therefore not representative of antibodies found in patients with APS.

A murine monoclonal anti-phosphorylcholine (PC) antibody 6G6 was expressed as whole IgM molecules secreted from HCLV (Pewzner-Jung et al., 1996). It was demonstrated that both the light chain and CDR3 of the heavy chain were necessary for binding to PC. A later study expressed a number of human antibodies in the form of Fab produced by *E.coli*

(Kumar et al., 2000). These experiments showed that the ability to bind CL was mainly dependent on the L chain of some human antibodies, but the H chain of others. These studies, however, were primarily directed at investigating the origin and properties of anti-DNA Ab. Neither group studied antibodies that were known to be relevant to APS on the basis of β_2GPI dependent binding to PL or pathogenicity in mouse models. Indeed the first report of *in-vitro* expression of whole human aPL antibodies with these pathogenic properties has stemmed from work described in this thesis (Giles et al., 2003a).

1.10.5 Work which needs to be carried out

There remains a need to investigate sequence related binding and pathogenic properties of a human aPL that are representative of those antibodies most closely related to disease pathogenesis in patients with APS. A monoclonal antibody of this type would need to be IgG isotype, bind to known to β_2GPI and be pathogenic *in-vivo*. Two such antibodies are IS4 and CL24 derived from two APS patients (Zhu et al., 1999). Both IS4 and CL24 possess multiple features likely to make them relevant to the pathogenesis of APS. These aPL were derived from a patient with PAPS (IS4) and an SLE/APS patient (CL24). They are of IgG isotype, bind with high affinity to anionic PL only in the presence of β_2GPI and can bind to β_2GPI alone. Both antibodies have been shown to be thrombogenic in a murine *in-vivo* pinch-induced thrombosis model and to cause EC activation *in-vitro* (IS4 and CL24) and *in-vivo* (IS4 only) (Pierangeli et al., 2000). The properties of the antibodies IS4 and CL24 are therefore likely to be relevant to the properties of pathogenic aPL found in many APS patients.

Therefore I chose to examine which sequence features in IS4 and CL24 H and L chains were important in determining their ability to bind antigen by expressing whole antibodies using a mammalian/eukaryotic system. Experiments to produce variant forms of antibodies using heavy/light chain exchange, CDR exchange and site directed mutagenesis were also carried out and their effects upon antibody binding to phospholipid examined. Although the yield from eukaryotic systems is smaller than with bacterial systems, the whole antibody produced is capable of bivalent binding to β_2GPI (Rahman et al., 2001) and can potentially be used in assays of pathogenicity which is a major advantage over antibody fragments produced from bacterial expression systems.

1.11 AIMS OF THIS THESIS

1. To determine and analyse the sequence of the V_H and V_L regions of two human monoclonal IgG anti-ß$_2$GPI antibodies (IS4 and CL24) by cloning variable region cDNA into pGEM-T Easy Vector for manual sequencing and analysis.

2. To determine the relative contribution of sequence features in the H and L chains of IS4 and CL24, expressed as whole IgG molecules in COS-7 cells, in their ability to bind CL.

3. To make variant forms of each V region with altered patterns of mutation or basic residues by CDR exchange between 2a2 derived light chains and site directed mutagenesis of IS4V_H and examine the CL binding properties of these variants.

4. To develop a system for the stable expression of cloned antibody DNA sequences, derived from human monoclonal aPL and anti-dsDNA mAb, as whole IgG molecules in Chinese hamster ovary (CHO) cells which lack the dihydrofolate reductase (*dhfr*) gene.

CHAPTER 2 MATERIALS AND METHODS

2.1 Laboratory Reagents

Chemicals, general materials and equipment are listed in appendix B whilst enzymes, buffers and solutions are listed in appendix C.

2.2 Plasmids and oligonucleotides

The pGEM-T Easy vector (Promega, Southampton, U.K.) insertion site contains single 3`dT overhangs on both sides, which are compatible with the single 3`dA base produced by the action of *Taq* polymerase thus allowing rapid cloning of purified PCR products.

Vectors pBCVHCASS4, pG1D1, pG1D106, pG1D210, pKN100, pLN10, pLN100 were all kind gifts from Dr C.A. Kettleborough and Dr T. Jones at AERES Biomedical, Mill Hill, London.

The intermediate expression vector pBCVHCASS4 shown in Figure 2.1 is based upon the Stratagene phagemid vector pBCSK+ containing a chloramphenicol resistance gene. pBCVHCASS4 vector was created from pBCSK+ by insertion of a cassette containing a eukaryotic leader sequence together with a number of restriction sites. The leader sequence is situated such that it will be in frame with a variable region gene inserted between the *Sfi* I and *Bam*H I sites at position 742 and 811 (see Figure 2.1).

The final expression vectors pG1D1, pG1D106, pG1D210, pKN100, pLN10 and pLN100 shown in Figures 2.2 – 2.7, all contain a human cytomegalovirus (HCMV) promoter to drive transcription of a recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and an ampicillin resistance gene driven by an internal promoter to enable it to be expressed in *E.coli*. The vectors also contain selectable genes (for dihydrofolate reductase or for neomycin resistance) which enable them to be selected in a eukaryotic expression system. In pG1D106, pG1D210, pKN100, and pLN100 vectors the CMV IEI promoter contains a regulatory sequence intron A, which has been shown to increase the levels of expression of glycoprotein from vectors containing this additional sequence (Chapman et al., 1991). Plasmids pG1D1 and pLN10 are "old" versions of pG1D210 and pLN100 respectively which lack intron A.

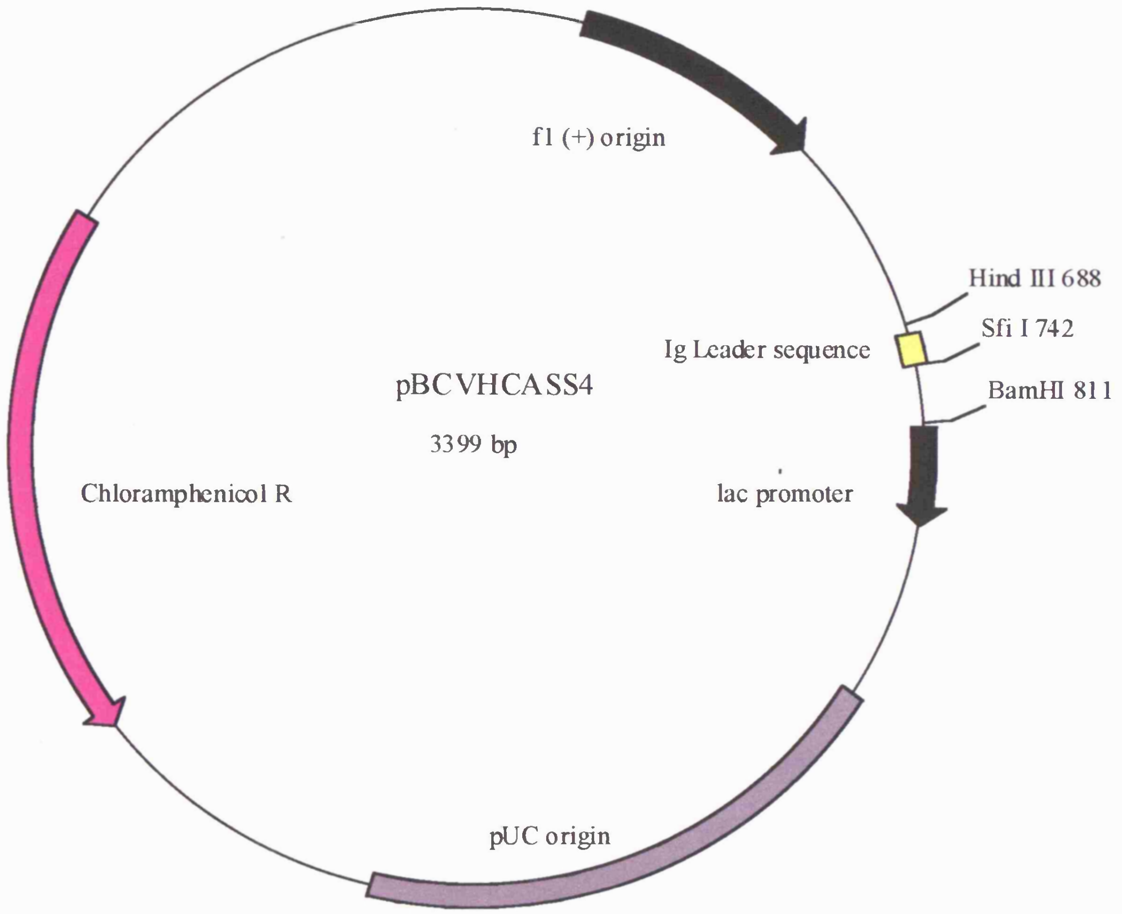


Figure 2.1 Plasmid map of intermediate expression vector pBCVHCASS4

Abbreviations:- Chloramphenicol R - chloramphenicol resistance gene; Ig Leader sequence – immunoglobulin eukaryotic leader sequence.

pG1D1 and pG1D106 (Figures 2.6 and 2.2 respectively) contain a human cDNA version of the gamma-1 constant region (Human C_H), 5` to which is a multiple cloning site (MCS) allowing the insertion of a variable domain gene immediately followed by a splice donor site. The human C_H is immediately preceded by a splice acceptor site hence the DNA between the V domain and C region is treated like a V::C intron by mammalian cells. The C_H is followed by a termination sequence to prevent read through. The promoter driving the dihydrofolate reductase (*dhfr*) gene is crippled so that expression is poor making selection by methotrexate amplification during stable transfection possible. pG1D210 (Figure 2.3) is identical to pG1D106 save for the removal of the V::C intron and the replacement of the MCS with a new sequence which also encodes the last few amino acids of a heavy chain J region, i.e. GTLVTVSS. V_HJ_H sequences can be cloned into the vector directly adjacent to C_H and in frame with it.

The light chain expression vectors pLN10 and pLN100 (Figures 2.7 and 2.5 respectively) both contain a human cDNA version of the λ-2 constant region sequence DNA (C_λ) whilst pKN100 (Figure 2.4) contains human κ constant region sequence cDNA (C_κ). In all three vectors 5` to the constant region cDNA (human C_L) is a MCS allowing the insertion of a variable domain gene immediately followed by a splice donor site. The constant region cDNA is immediately preceded by a splice acceptor site hence the DNA between the V domain and C region is treated like a V::C intron by mammalian cells and not represented in the expressed light chain peptide. A termination sequence follows the human C_L to prevent read through. The bacterial neomycin resistance gene (NeoR) may be used as a dominant selectable marker during stable transformation.

Recombinant expression vectors; pG1D1 containing human immunoglobulin V_H cDNA and pLN10 containing human immunoglobulin V_L cDNA were constructed by Dr Anisur Rahman, shown in Figures 2.6 - 2.7: and pG1D210 containing human immunoglobulin V_H cDNA was constructed by Dr Joanna Haley, shown in Figure 2.8.

All oligonucleotide primers were supplied by Genosys, Cambridge, U.K.

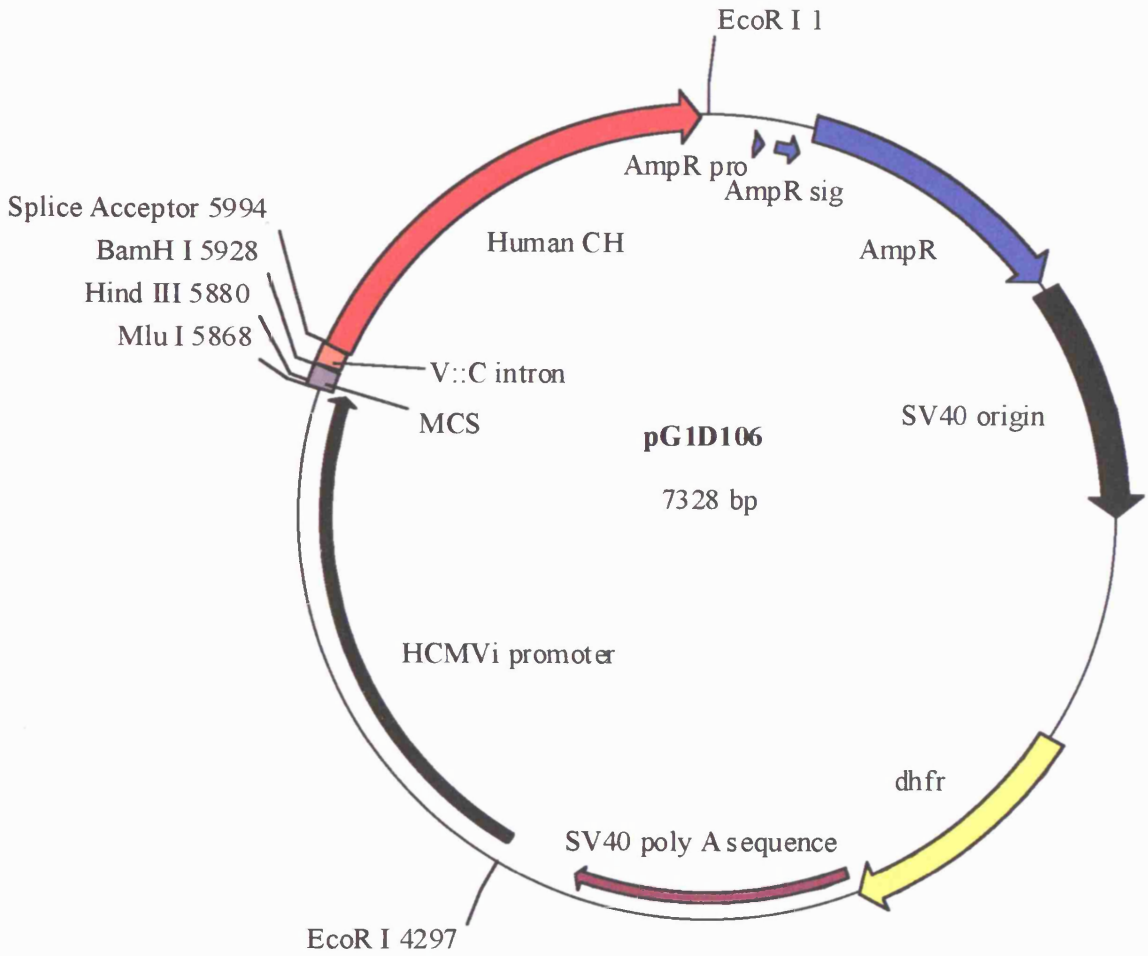


Figure 2.2 Plasmid map of expression vector pG1D106

Abbreviations: - HCMVi – improved human cytomegalovirus promoter containing intron A; Human CH - cDNA version of the gamma-1 constant region; MCS - multiple cloning site; V::C intron - region of plasmid DNA between variable (V) domain insert and constant (C) region treated like a V::C intron by mammalian cells; AmpR pro – promoter for ampicillin resistance gene; AmpR sig – signal sequence for ampicillin resistance gene; AmpR - Ampicillin resistance gene; dhfr - dihydrofolate reductase gene.

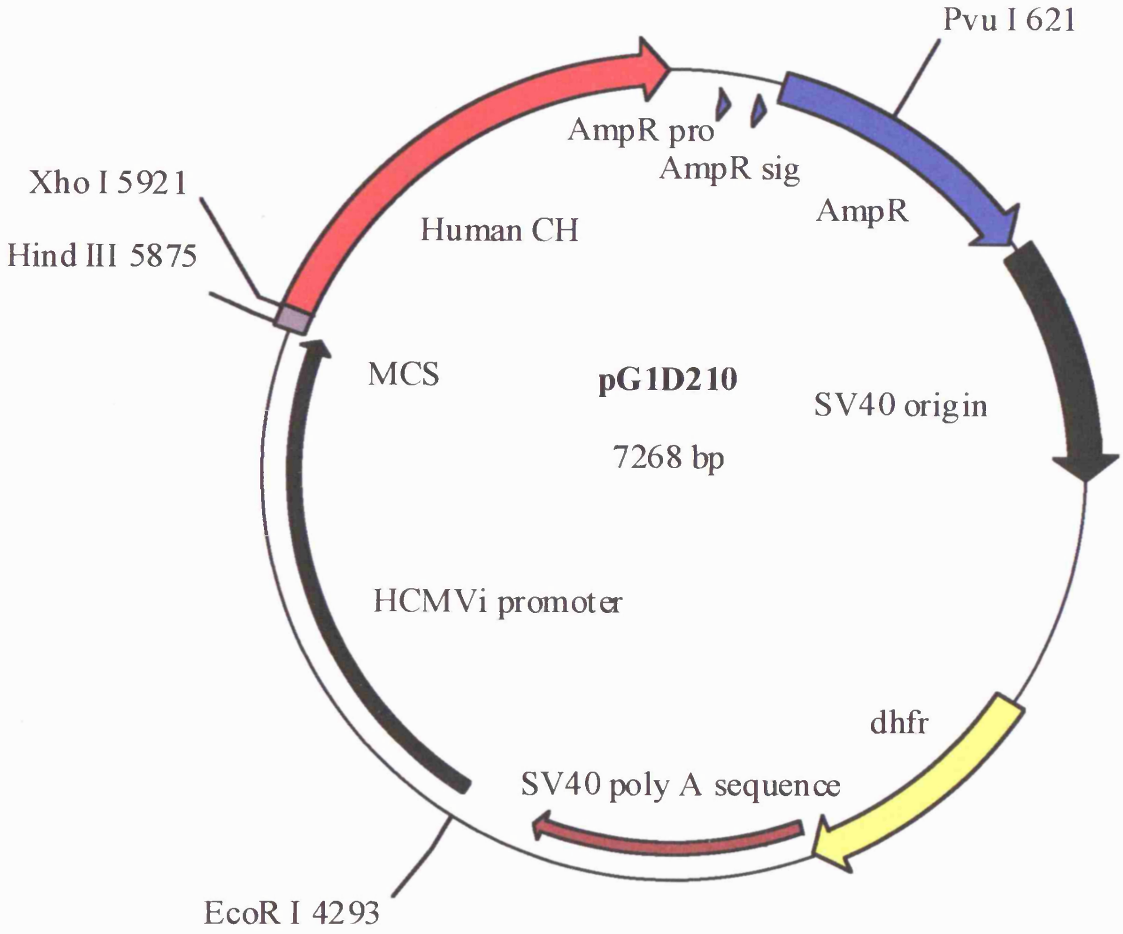


Figure 2.3 Plasmid map of expression vector pG1D210

Abbreviations: - HCMVi – improved human cytomegalovirus promoter containing intron A; Human CH - cDNA version of the gamma-1 constant region; MCS - multiple cloning site; AmpR pro – ampicillin internal promoter; AmpR sig – ampicillin signal sequence; AmpR - Ampicillin resistance gene; dhfr - dihydrofolate reductase gene.

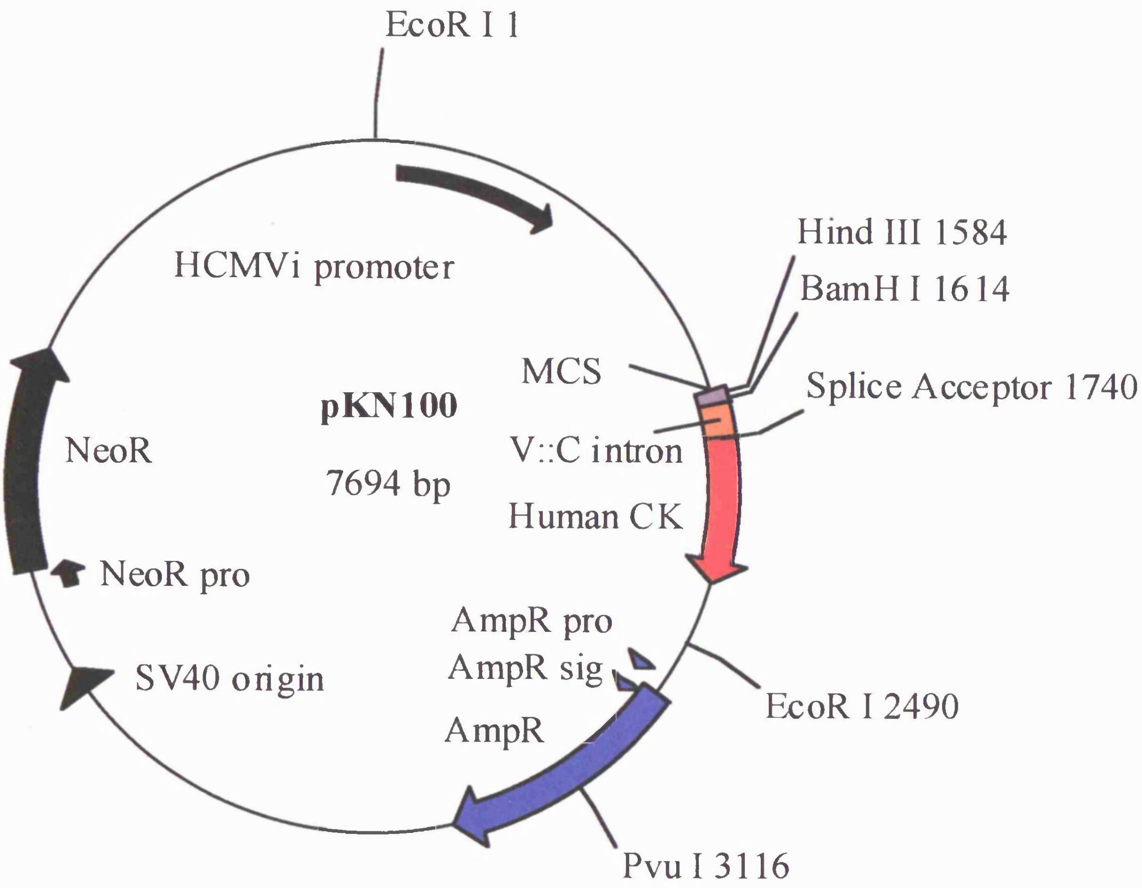


Figure 2.4 Plasmid map of expression vector pKN100

Abbreviations: - HCMVi – improved human cytomegalovirus promoter containing intron A; Human CK - cDNA version of the human (allotype Km3) constant region; MCS - multiple cloning site; V::C intron - region of plasmid DNA between variable (V) domain insert and constant (C) region treated like a V::C intron by mammalian cells; AmpR pro – promoter for ampicillin resistance gene; AmpR sig – signal sequence for ampicillin resistance gene; AmpR - ampicillin resistance gene; NeoR pro – promoter for neomycin resistance gene; NeoR – neomycin resistance gene.

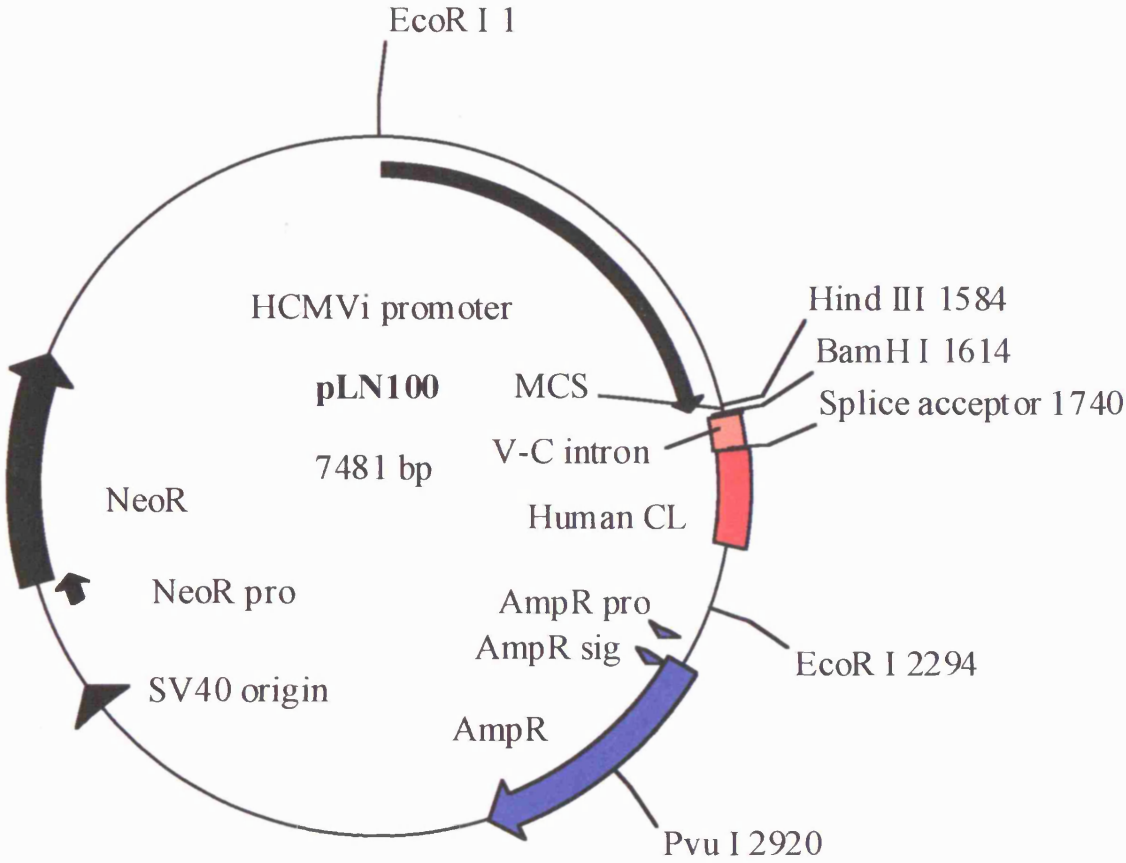


Figure 2.5 Plasmid map of expression vector pLN100

Abbreviations: - HCMVi – improved human cytomegalovirus promoter containing intron A; Human CL - cDNA version of the human lambda-2 constant region; MCS - multiple cloning site; V::C intron - region of plasmid DNA between variable (V) domain insert and constant (C) region treated like a V::C intron by mammalian cells; AmpR pro – promoter for ampicillin resistance gene; AmpR sig – signal sequence for ampicillin resistance gene; AmpR - ampicillin resistance gene; NeoR pro – promoter for neomycin resistance gene; NeoR – neomycin resistance gene.

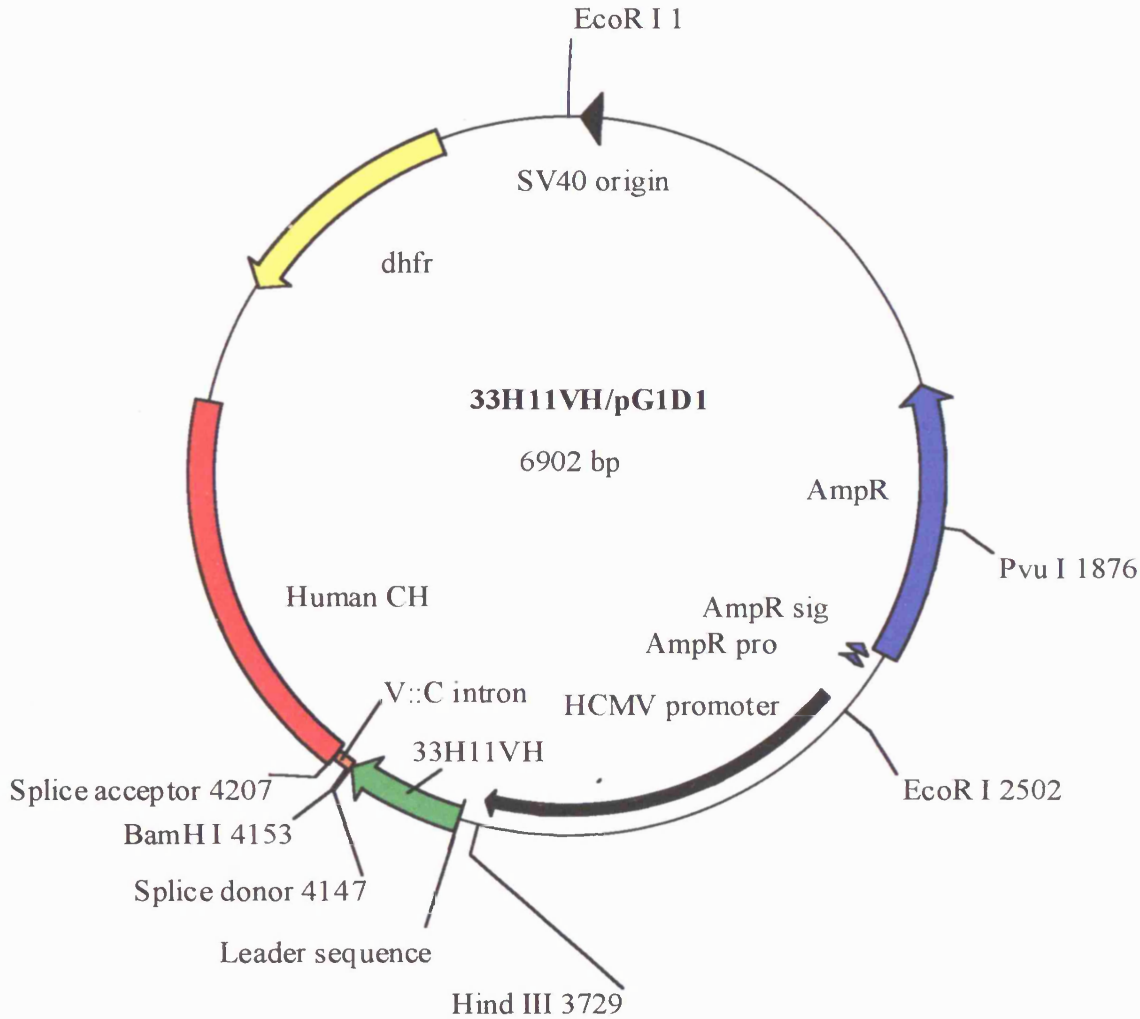


Figure 2.6 Vector map of recombinant heavy chain expression vector 33H11V$_H$/pG1D1

This vector containing the V$_H$ region of 33H11 (including a leader sequence from pBCVHCASS4) had been previously cloned by Dr A. Rahman.

Abbreviations: - HCMV – human cytomegalovirus promoter; Human CH - cDNA version of the gamma-1 constant region; MCS - multiple cloning site; V::C intron - region of plasmid DNA between variable (V) domain insert (33H11 V$_H$) and constant (C) region treated like a V::C intron by mammalian cells; AmpR pro – promoter for ampicillin resistance gene; AmpR sig – signal sequence for ampicillin resistance gene; AmpR - Ampicillin resistance gene; dhfr - dihydrofolate reductase gene.

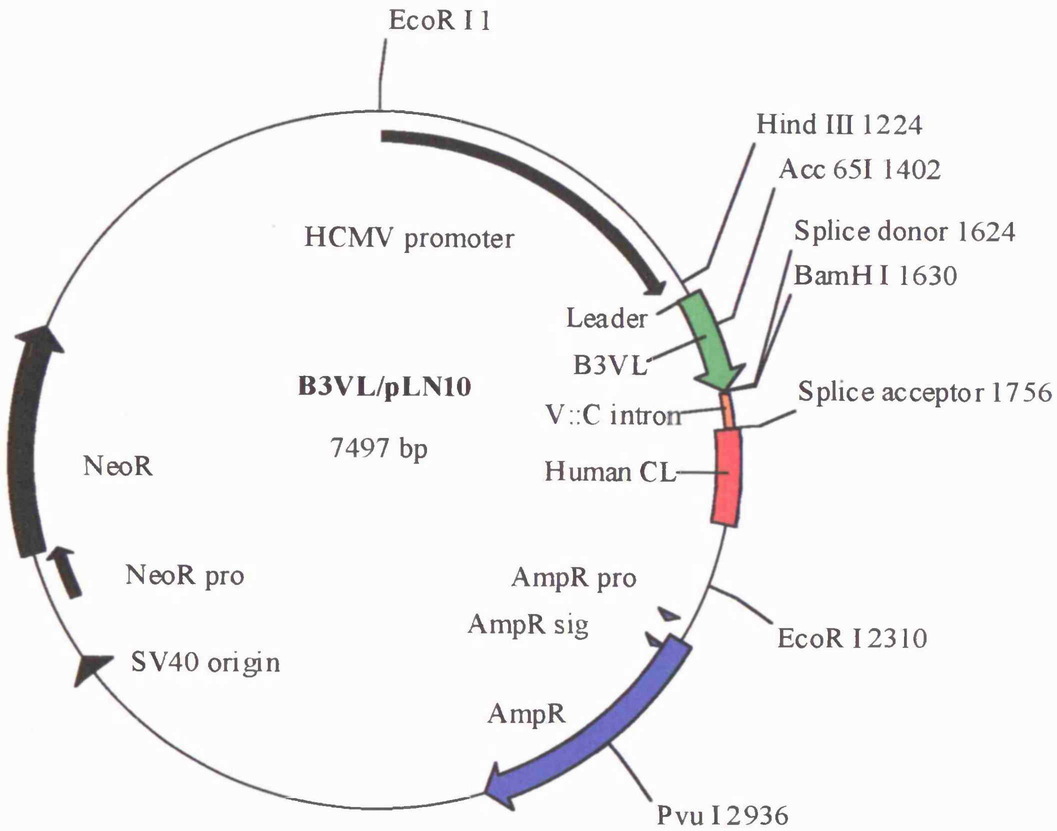


Figure 2.7 Vector map of expression vector B3V_L/pLN10

The V_L region of B3 (including a leader sequence from pBCVHCASS4) had been previously cloned by Dr A. Rahman into pLN10. The vector map of 33H11 V_L /pLN10 is identical to that shown above but lacks an *Acc*65 I site in 33H11V_L. UK4V_L is six nucleotides smaller than B3V_L in CDR3, hence the features shown above are the same for UK4V_L/pLN10 to the *Acc*65 I site after which the position of all regions and sites is six nucleotides less than in B3V_L/pLN10.

Abbreviations: - HCMV – human cytomegalovirus promoter; Human CL - cDNA version of the human lambda-2 constant region; MCS - multiple cloning site; V::C intron - region of plasmid DNA between variable (V) domain insert (B3VL) and constant (C) region treated like a V::C intron by mammalian cells; AmpR pro – promoter for ampicillin resistance gene; AmpR sig – signal sequence for ampicillin resistance gene; AmpR - ampicillin resistance gene; NeoR pro – promoter for neomycin resistance gene; NeoR – neomycin resistance gene.

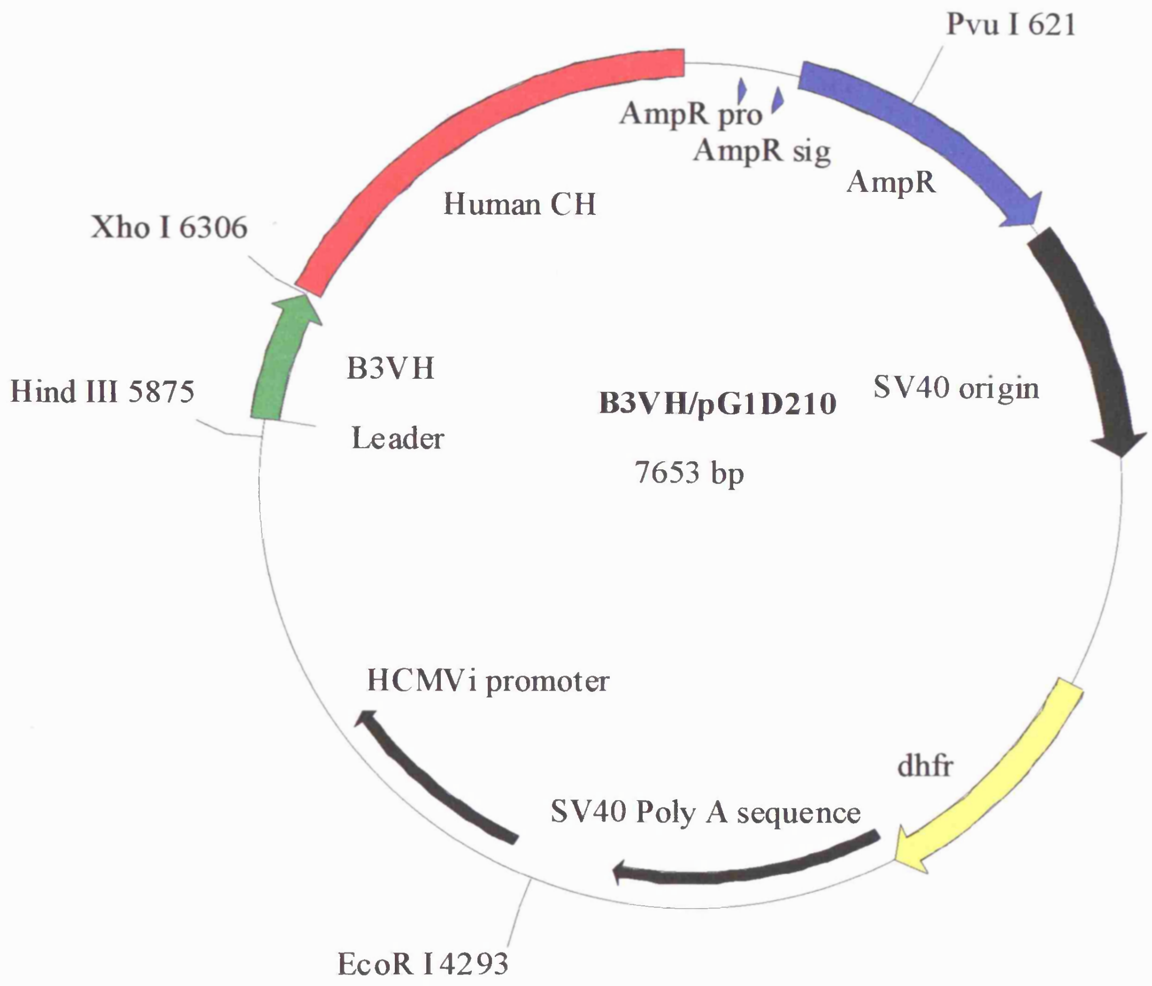


Figure 2.8 Vector map of recombinant heavy chain expression vector B3V$_H$/pG1D210

The V$_H$ region of B3 (including a leader sequence from pBCVHCASS4) had previously been cloned into pG1D210 by Dr J. Haley.

Abbreviations: - HCMVi – improved human cytomegalovirus promoter containing intron A; Human CH - cDNA version of the gamma-1 constant region; MCS - multiple cloning site; AmpR pro – promoter for ampicillin resistance gene; AmpR sig – signal sequence for ampicillin resistance gene; AmpR - Ampicillin resistance gene; dhfr - dihydrofolate reductase gene.

2.3 Bacterial Strains

Escherichia coli of strain DH5α	Invitrogen, Paisley, U.K.
Escherichia coli XL1-Blue Supercompetent cells	Stratagene, California, USA.

2.4 Human monoclonal antibodies

IS4, CL24, B3, 33H11 and UK4 are all human IgG monoclonal antibodies produced from lymphocytes of five different patients. IS4 and CL24 were derived from patients with PAPS and SLE/APS respectively by EBV transformation of peripheral blood mononuclear cells and fusion with the human-mouse heterohybridoma K6H6/B5 cell line (Zhu et al., 1999). Both IS4 and CL24 bind to CL in the presence of bovine and human β_2GPI and to human β_2GPI alone (Zhu et al., 1999). B3 (Ehrenstein et al., 1994), 33H11 (Winkler et al., 1992) and UK4 (Menon et al., 1997) were isolated by fusion of PBL from patients with cells of the mouse human heteromyeloma line CB-F7. 33H11 is specific for dsDNA, B3 binds ssDNA, dsDNA and histones (Ehrenstein et al., 1994; Kalsi et al., 1996). UK4 binds negatively charged (but not neutral) PL in the absence of β_2GPI and does not bind DNA (Menon et al., 1997). The hybridoma cell lines of CL24 and IS4 were all produced and kindly donated by Dr P.Chen, Department of Medicine, Division of Rheumatology, University of California at Los Angeles, Los Angeles, USA and maintained by Dr Jose Alves at the Centre for Rheumatology, University College London (U.C.L.), U.K. 33H11 (Winkler et al., 1992) was a kind gift from Dr Thomas Winkler (Erlangen, Germany). B3 (Ehrenstein et al., 1994) and UK4 (Menon et al., 1997) were produced by our group at U.C.L. in London. The characteristics of the patients are shown in table 2.1 and original antibody binding properties of IS4 and CL24 displayed in table 2.2.

2.5 Extraction of total RNA from hybridoma cells

2.5.1 Counting cells using a haemocytometer

Working under sterile conditions 0.2mls of hybridoma cell suspension was placed into a sterile bijou and diluted with 0.5mls of 0.4% Trypan blue solution and 0.3mls of Hanks Balanced Salt Solution (HBSS) then allowed to stand for five minutes. Trypan blue stains only dead (non-viable) cells blue, thus enabling the number of viable (white) cells to be counted. With the cover slip in place, enough of the cell suspension was transferred to fill both chambers of a sterile haemocytometer.

Feature	Patient 1	Patient 2	K.H.	D.H.	SLE2
Thrombosis	+	+	+	-	NS
Pulmonary Emboli	-	+	-	-	NS
CNS	+	NS	-	+	NS
Miscarriages	-	NS	-	-	NS
Migraines	NS	NS	-	+	NS
Rash	-	NS	+	-	NS
Arthritis	-	NS	+	+	NS
Serositis	NS	NS	-	+	NS
Thrombocytopenia	NS	NS	-	-	NS
Renal Disease	-	+	-	+	+
ANA	+	+	+	+	NS
dsDNA	-	+	+	+	+
Decreased C3 & C4	-	+	-	+	+
LA	+	+	-	-	NS
aCL	+	+	+	+	NS

Table 2.1 Clinical and serological characteristics of patients from whom monoclonal antibodies were derived

IS4 was derived from PBL of a 19 year old patient with **PAPS** (patient 1). CL24 was derived from PBL of a 17 year old **SLE/APS patient** (patient 2). B3 was derived from PBL of a 60 year old patient with SLE (K.H.). UK4 was derived from PBL of a 49 year old patient with SLE (D.H.). 33H11 was derived from PBL of a SLE patient (SLE2). NS indicates where it was not stated in the relevant journal paper whether the patient from whom a mAb was derived, had the particular feature or not.

	IS4	CL24
Immunological reactivity		
Antigens		
CL/BS	++	++
CL alone	-	-
CL/HS	+	+
CL/human B_2GPI	++	++
Human B_2GPI alone	+	+
Functional reactivity		
LA	-	+

Table 2.2 Immunological characteristics of IS4 and CL24 monoclonal aPL

Abbreviations: - CL - Cardiolipin; BS - Bovine Serum; HS - Human Serum; LA – Lupus Anticoagulant.
Modified from Table II, Zhu *et al.* (1999).

Under the light microscope the number of viable cells in the central 1mm square and the four corner 1mm squares was counted in chamber one and an average obtained. Only cells completely inside a square and those touching the right and bottom perimeter of each square were counted. The procedure was repeated for chamber two. Each chamber of the haemocytometer with cover slip in place represents a total volume of $0.1mm^3$. The final cell count was taken as an average from the cell count for each chamber. The total numbers of cells were calculated using the following formula:-

Cell count (average) x Dilution Factor (5) x 10^4 x original volume of fluid from which cell sample was removed.

The total number of cells recovered was approximately 10^6 to 10^7.

2.5.2 Isolation of RNA

This was achieved using RNAzol B, which contains phenol and guanidium thiocyanate. A suspension of approximately 10^6 to 10^7 hybridoma cells was centrifuged for 10 minutes at 250g at 4°C. Supernatant was discarded and the cell pellet resuspended in 20 ml PBS then centrifuged at 250g at 4°C for five minutes. Supernatant was removed by aspiration and 200μl of RNAzol per million cells added to the cell pellet, mixed thoroughly and then transferred to a sterile 0.5 ml microfuge tube. A 1/10 volume of chloroform was added, vortexed for 15 seconds and then the tube was left on ice for 15 minutes. The suspension was spun at 13000g in a microcentrifuge for 15 minutes and the upper aqueous layer containing RNA transferred to a fresh 0.5 ml microfuge tube. An equal volume of propan-2-ol was added and the mixture kept at -20°C for at least one hour (or overnight) to precipitate the RNA. Following this a small pellet of RNA was recovered by centrifugation at 13000g for 15 minutes and then "washed" by adding 1 ml of 75% ethanol in 0.1% DEPC water. In this context "washed" means that the pellet was resuspended in 75% ethanol, then reprecipitated by centrifugation at 3300g for 10 minutes. Supernatant was completely removed and the pellet was dried at 37°C for approximately 30 minutes. Finally 10μl of 0.1% DEPC water was added in which the RNA was fully dissolved by incubation at 65°C for 10 minutes before being stored at -80°C.

2.6 Synthesis of cDNA from hybridoma total RNA

A 1µl aliquot of stored RNA containing approximately 1µg of hybridoma total RNA was removed from storage and heated at 65°C for two minutes before being transferred to a 0.5ml microfuge tube. The following reaction mixture was then added; 2µl 25mM $MgCl_2$, 2µl of a mixture of dATP, dCTP, dGTPand dTTP containing each dNTP at a concentration of 25mM, 1µl random hexamers (0.9 units/ml), 2µl 10xPCR Buffer, 0.5µl (40u/µl) ribonuclease inhibitor (RNAsin), 0.5µl (200u/µl) AMV reverse transcriptase and autoclaved ddH_20 to 20µl.

Each mixture was pulse spun at 13000g in a bench centrifuge and then placed in a 37°C bath with the closed lid covered with parafilm for one hour. The products were then used immediately as the substrate in a PCR.

2.7 Amplification of V_H and V_L cDNA by PCR

In this step only cDNA encoding the V region of IS4 and CL24 produced by the hybridoma cells from which RNA had been extracted is selectively amplified by specific PCR primers (see Table 2.3).The following reaction mixture was added to the 20µl cDNA from section 2.6; 10µl 10xPCR Buffer, 8µl 25mM $MgCl_2$, 1µl of a mixture of dATP, dCTP, dGTPand dTTP containing each dNTP at a concentration of 25mM, 1µl forward primer (final concentration 1µM), 1µl reverse primer (final concentration 1µM), autoclaved ddH_2O to 80µl and 1µl (5u/µl) *Taq* Polymerase.

As a negative control no cDNA was added to one reaction mixture. Each mixture was then pulse spun and placed into a PCR machine programmed as follows:-

Initial denaturation at 94°C for 5 minutes

Then 30 cycles as follows: –

94°C for 1 minute

Annealing temperature for one minute -	65°C for V_κ primers
	55°C for V_λ primers
	55°C for VH1 and VH3 primers
	42°C for VH2, VH5 and VH9 primers

72°C for one minute

Then a final elongation phase at 72°C for 5 minutes.

The presence of PCR product of the expected length (350 – 400bp) was confirmed by gel electrophoresis through a 1% agarose gel (see section 2.8).

PCR Primers used in this step are shown in table 2.3. The forward primer JL was used to amplify IS4 V_λ region and binds to the 3` terminus of J_λ (Hawkins et al., 1994). Amplification of each V_H region was achieved using a C_γ forward primer that binds to a six codon stretch of the gamma constant gene about 20-30bp from the J_HC_γ junction (Stevenson et al., 1993). Similarly the forward primer CK was used to amplify CL24 V_κ (Stevenson et al., 1993).

Primers	Sequences
HL 10	5`CAGTCTGTGTTGACGCAGCCGCCCTC3`
HL 20	5`CAGTCTGCCCTGACTCAGCCTGCCTC3`
HL 30	5`TCCTATGAGCTGACTCAGCCACCCTC3`
JL	5`ACCTAGGACGGTCAGCTTGGTCCC3`
VK 1	5`GACATCCAGATGACCCAGTCTCC3`
VK 2	5`GATATTGTGATGACTCAGTCTCC3`
VK 3	5`GAAATTGTGTTGACGCAGTCTCC3`
CK	5`CGAGAATTCTAGATCATCAGATGGCGGA3`
VH 2	5`CAGGTGCAGCTGGTGGAGTCTGG3`
VH 4	5`ATGAAACACCTGTGGTTCTT3`
VH 5	5`GAGGTGCAGCTGCTGCAGTCTG3`
VH 1	5`CAGGTCCAGTTTGTGCAGTCT3`
VH 3	5`GAAGTGGAGTTATTGGAATCT3`
Cγ	5`CAGGGGGAAGACCGATGG3`

Table 2.3 PCR primers used to amplify V_H and V_L cDNA for initial sequencing

Back primers HL10, HL20 and HL30 which bind to the first nine codons of genes belonging to the $V_\lambda 1$, $V_\lambda 2$ and $V_\lambda 3$ families respectively were used to amplify and identify IS4 V_λ (Hawkins et al., 1994). To identify CL24 V_κ the VK1, VK2 and VK3 primers which bind to the first eight codons of genes from $V_\kappa 1$, $V_\kappa 2$ and $V_\kappa 3$ families respectively were used (Hawkins et al., 1994). Initially the back primers VH2, VH5 and VH9 were employed to identify and amplify the V_H regions. VH2 and VH5 primers bind specifically to the first seven codons of genes from V_H2 and V_H5 families respectively whilst the VH9 primer is designed to show homology to the first eight codons of the V_H gene segment of families V_H1, V_H3 and V_H4 (Rahman, 1998). Later the sequences of IS4 and CL24 were provided by Dr Chen so primers could be designed which bound specifically to the first seven codons of IS4 and CL24 V_H. These primers were designated VH3 and VH1 respectively.

2.8 Separation of DNA fragments by agarose gel electrophoresis

One litre of TAE buffer was prepared (see appendix C), 100mls of which were then removed and mixed with 1g of agarose, or low melting point agarose (Invitrogen), to make a 1% gel. The solution was heated in a microwave oven until all the agarose had dissolved and then allowed to cool until hand hot when 2μl of 10mg/ml ethidium bromide was added. Ethidium bromide molecules intercalate between base pairs in the DNA double helix rendering it fluorescent when viewed under ultraviolet (UV) light.

The open ends of a gel tray (Bio-Rad) were closed off with tape and the agarose solution poured in. Plastic combs were then placed in the gel tray to create wells in the agarose as it solidified and any air bubbles were removed. Once the gel had set the comb and tape were carefully removed and the gel tray placed in an electrophoresis tank (Bio-Rad) to which the remaining 900mls of TAE buffer was added.

To each 12μl of sample to be loaded onto the gel 2μl of loading buffer were added. This buffer contains Ficoll which increases the density of the sample, ensuring the DNA in each sample sinks evenly into the well. The buffer also colours the sample with a dye (xylene cyanole) that moves towards the anode at a predictable rate, allowing progress through the gel to be monitored. Between 10 and 40μl of each sample were then added to each well and a voltage of 100 Volts applied for one hour.

The gel was then removed from the electrophoresis tank and examined under UV light. The sizes of bands in the gel were estimated by comparison with a standard DNA molecular weight marker loaded into another well in the same gel. Photographs of the gel were taken using the Genesnap programme (Syngene, Cambridge, UK) and printed onto paper. If the DNA had to be recovered from the gel the appropriate band was cut out with a scalpel and stored at -20°C.

The concentration of each DNA fragment was estimated by running the product with molecular weight markers. The latter contained a known concentration of DNA. Thus comparison of the relative brightness of each band produced enabled the estimation of the concentration of DNA upon the gel and thus by extrapolation in the total sample.

2.9 Purification of amplified V_H and V_L cDNA from agarose gels

DNA excised from agarose gels was initially purified by adsorption onto silica particles (method A) using the Geneclean II kit (Anachem, Luton, UK) but the QIAquick gel extraction (Qiagen, Crawley, West, Sussex, U.K.) kits were later found to increase the yield of recombinant DNA (method B).

2.9.1 Method A

Each excised band of DNA was placed in a 1.5ml microfuge tube and its volume determined by approximating 0.1g of the gel slice to equal 100µl. Three times the volume of sodium iodide (NaI) was added and the agarose melted by incubation in a 55°C water bath for five minutes. 5µl of glassmilk were added per 500µl of final volume of solution unless the final volume exceeded 1ml in which case 20µl of glassmilk were used. Following this the mixture was incubated at room temperature for five minutes with regular mixing by vortex every 1-2 minutes. The mixture was then spun at 13000g for 5 seconds and the supernatant removed and saved in case all of the DNA did not bind to the glassmilk. The pellet was washed three times in 500µl of "New Wash" buffer supplied in the kit (see appendix C). Once all of the supernatant from the third wash had been removed the pellet was dried under a vacuum for approximately three minutes and resuspended in a volume of water equal to the amount of glassmilk used. The suspension was then centrifuged at 13000g for one minute and supernatant containing eluted DNA carefully removed and placed in a fresh 1.5ml microfuge tube. A second elution was carried out to recover a further 10-20% of DNA and saved with the product of the first elution at -20°C.

2.9.2 Method B

An excised band of DNA of up to 0.4 g was placed in a 1.5ml microfuge tube and 1ml of buffer QG added. The mixture was then incubated at 55°C for 10 minutes until all agarose had dissolved. 700μl of this solution was then added to a spin column placed in a collection tube and centrifuged at 13,000g for 10 seconds. Flow through was discarded from the collection tube and the procedure repeated until all of the initial mixture had been passed through the spin column. A further 500μl of buffer QG was added to each spin column and centrifuged at 13,000g for 10 seconds. After discarding the flow through the column was washed twice with 500μl of buffer PE and centrifuged as above. Each column was then placed into a fresh 1.5ml microfuge tube and dried under a lamp for 5 minutes. Elution of DNA from each column was performed by addition of 50μl of solution EB (10mM Tris-HCl pH 8.5), allowing the column to stand for 1 minute followed by centrifugation as above. The product was then stored at -20°C.

2.10 Ligation of amplified cDNA into pGEM-T Easy Vector

Purified PCR products from step 2.9 were then ligated into (50ng) pGEM-T Easy Vector by TA cloning at a range of 3:1 to 1:3 insert:vector molar ratios. To 4μl of pGEM-T Easy Vector: purified PCR product mix, 1μl 10x ligase buffer, 4μl autoclaved water and 1μl (3u/μl) T4 DNA ligase were added. Samples were incubated overnight at 4°C. The ligation product was used to transform competent DH5α cells.

2.11 Production of competent DH5α cells

DH5α cells picked from a discrete colony upon an LB agar plate were grown overnight in 10mls of LB medium at 37°C in a shaking incubator (300rpm). The following morning 100μl of this culture were added to 100ml of LB in a sterile flask and returned to the shaking incubator for approximately three hours. When swirling clouds of bacteria were just visible the culture was removed from the incubator and divided into two 50ml centrifuge tubes which were then centrifuged at 1864g for 10 minutes at 4°C. Supernatant was discarded from each tube and the pellet resuspended in 10mls of ice cold 100mM

$CaCl_2.6H_2O$. Each tube was again centrifuged at 1864g for 10 minutes at 4°C and the supernatant discarded. Finally each pellet was resuspended in 2mls of ice-cold 100mM $CaCl_2.6H_2O$ and this suspension was used for transformations.

2.12 Transformation of competent DH5α cells with recombinant pGEM-T Easy Vector

5μl of recombinant plasmid DNA were added to 200μl of competent DH5α cells and left on ice for 30 minutes. A heat shock of 42°C for 90 seconds was then applied, following which the cells were incubated in 800μl of LB at 37°C for one hour. The cell suspension was then centrifuged at 700g for 10 minutes at room temperature; most of the supernatant was poured off and the pellet resuspended in the remaining supernatant. This suspension was then spread onto an LB agar plate containing 50μg/ml ampicillin, 30μl Xgal (20mg/ml) and 30μl IPTG (200mg/ml). After drying on the bench for 15 minutes the plates were then incubated at 37°C overnight and finally stored at 4°C.

Only cells that have taken up pGEM-T Easy Vector should produce colonies on the ampicillin containing plates because the vector contains a gene conferring resistance to ampicillin whilst DH5α cells are sensitive to this antibiotic. Colonies containing both pGEM-T Easy Vector and insert will produce white colonies because the insert disrupts the β galactosidase gene in pGEM-T Easy Vector. Cells that contain self-ligated vector will produce blue colonies, as the β galactosidase gene remains intact and will thus be induced by IPTG to digest its substrate X-gal. Growth and differentiation of white colonies from blue ones sometimes required several days' storage at 4°C. After this white colonies were picked from the plate into 5ml LB cultures containing 50μg/ml of ampicillin and kept overnight in a 37°C shaking incubator. The following day 0.5ml aliquots of the cultures produced were mixed with 0.5ml of sterile glycerol and stored at -20°C.

2.13 Small scale extraction of cloned cDNA from *E.coli* for sequencing

Purification of plasmid DNA from overnight cultures of *E.coli* in LB medium was carried out by alkaline lysis (Birnboim and Doly, 1979), separation into an organic and solvent phase and elution of DNA using the Qiagen Miniprep Kit and buffers (see appendix C). A bacterial colony was picked from an agar plate and incubated in 5ml of LB containing

50μg/ml of ampicillin overnight at 37°C. A bacterial pellet was obtained by centrifugation at 1864g for 10 minutes at 4°C and dried by inversion. The pellet was resuspended in 250μl of buffer P1 and transferred to a 0.5ml microfuge tube. Then 250μl of buffer P2 was added and the tube inverted six times. Following this 350μl of buffer N3 was added, mixed by inversion and then centrifuged for 10 minutes. All subsequent centrifugation steps were carried out at 13000g in a microcentrifuge for one minute at room temperature. Supernatant from this step was decanted into a QIAprep spin column and centrifuged. DNA bound to the QIAprep spin column was washed by adding 0.75ml of Buffer PE and centrifuged. Flow through was discarded and the column centrifuged. After placing the QIAprep spin column in a clean 1.5ml microfuge tube recombinant DNA was eluted by adding 50μl of autoclaved water to the centre of each QIAprep column which was left to stand for one minute before being centrifuged. Eluted DNA was then stored at -20°C.

2.14 Restriction enzyme digestion of plasmid DNA

An *Eco*R I digest confirmed success of the ligation of amplified cDNA into pGEM-T Easy Vector. 1μg of plasmid DNA (quantified by gel electrophoresis, section 2.8) was incubated with 10u *Eco*R I and buffer H (described below) at 37° for one hour and the product run upon an agarose gel (see section 2.8).

Small scale digests were carried out to characterise the structure of a newly isolated plasmid. For the majority of confirmation digests 1μg of plasmid DNA and 10 units of restriction enzyme were usually incubated in a total volume of 20μl for one hour with the appropriate buffer, at the temperature specified by the enzyme manufacturer to achieve full digestion. For double digests with enzymes that required incompatible buffering conditions, digestion was achieved sequentially with the enzyme requiring the lowest salt concentration used first. The solution was then supplemented with additional salts to achieve the correct conditions for the second enzyme which was then incubated appropriately. The recipe for a typical small scale digest (unless stated otherwise) would be as follows:

Plasmid DNA	5μl (1μg DNA)
Enzyme	1μl (10u/μl)
10x Buffer	2μl
ddH_20	to 20μl

2.15 Sequencing and analysis of cloned variable region cDNA

In order for sequencing to be carried out plasmid DNA must first be denatured. Fresh 2M NaOH, 2mM EDTA solution was prepared and 1/4 volume added to 45μl of miniprep plasmid DNA containing 8-9μg DNA, then left at room temperature for 15 minutes. A 1/10 volume of 3M sodium acetate (pH 5.2) and 1.5 volumes of 100% ice cold ethanol were added and left at -70°C for 30-45 minutes. The solution was then centrifuged at 13000g for 10 minutes at 4°C and the DNA pellet washed with 100μl of ice cold 70% ethanol. The pellet was dried under a vacuum for 3-5 minutes and resuspended in 10-15μl of autoclaved water, then stored at -20°C.

The dideoxynucleotide chain termination method (Sanger et al., 1977) was performed using the Sequenase Version2.0 kit (USB). 7μl of denatured template DNA were added to 2μl of 5xSequenase Buffer and 1μl of the appropriate primer at a concentration of 1μM. This mixture was incubated at room temperature for 15 minutes so the primer could anneal to the template DNA. Primers used for sequencing insert in pGEM-T Easy Vector were the T7 promoter and SP6 which occur on either side of the inserted cDNA. During this incubation the termination 0.5ml microfuge tubes were labelled in sets of four with the name of the template/primer mix and the letter A, C, G or T on each. Then 2.5μl of a different dideoxyNTP (ddNTP, 8μM) was added to each tube. Thus each tube A contained ddATP, tube C ddCTP, and so on. Each "termination" tube was then placed in a 49°C water bath.

The following components were then added to the template/primer mix: - 0.7μl ^{35}SdATP, 1μl Dithiothreitol (DTT), 2μl of a labelling mix containing 1.5μM deaza-dGTP and 1.5μM each of dCTP and dTTP and 2μl (13u/μl) Sequenase (DNA Polymerase) diluted 1:7 in 10mM Tris HCl, 5mM DTT.

The reaction mixture was then incubated for two minutes at room temperature to enable elongation of complementary DNA strands (radiolabelled with ^{35}SdATP) from the primer, by DNA Polymerase. Following this 3.3μl of each reaction mixture were added to each of the four termination tubes and incubated at 49°C for 5 minutes. The sequencing reaction in each tube was then stopped by the addition of a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole. The incorporation in each termination tube of the respective ddNTP which lacks the terminal 3` hydroxyl group for chain extension means that the species of DNA of different lengths in tube A all end in ddATP, DNA in tube C ends in ddCTP and so on.

2.15.1 Polyacrylamide gel electrophoresis

Fresh sequencing gel solution (see appendix C) was prepared as above then drawn up into a 20ml syringe and expelled between two large glass sequencing plates. The plates were separated by plastic spacers at each side, two upside down plastic gel combs at the top and held together by bulldog clips. Fluid was drawn between the plates by capillary action and frequent tapping of the plates at the leading gel edge with a constant supply of fluid at the top of the plates ensured an even spread of the gel with no air bubbles. The gel was left for one hour to solidify as a thin sheet of polyacrylamide between the plates. Following this the plates were placed vertically with the comb facing inwards in an electrophoresis bath (Invitrogen) containing 1l of TBE buffer (see appendix C). The combs were then reversed so the shark tooth edge pierced the gel creating 48 wells. Samples from sequencing reactions were denatured by boiling at 100°C for two minutes and then 3μl loaded such that A, C, G, T tubes from the same reaction were in adjacent wells.

A current was passed through the gel at a power of 42W for approximately 2 hours and twenty minutes. The plates were removed and the gel transferred to 3MM chromatography paper (Whatman International Ltd, Maidstone, U.K.) then covered in cling-film and dried in a vacuum drier for 1-3 hours. Following this the clingfilm was removed. In a dark room the gel was placed in an autorad cassette (Bio-Rad) with Kodak (X-OMAT) AR film (Amersham Pharmacia Biotech, Bucks, U.K.) and the cassette tightly sealed. Typical exposure of the film was for 2-5 days, before developing (Xograph, Bedfordshire, U.K.) and reading the sequence of dark bands representing chains of DNA of different length.

2.15.2 Alignment to closest possible germline alleles

Germline genes of origin for each V_H and V_L chain of IS4 and CL24 were obtained using DNAplot software in VBASE, which is a comprehensive sequence directory of all known human Ig genes and their alleles (Tomlinson et al., 1996). Nucleotide homology was then calculated using the following formula and expressed as a percentage.

$$\frac{\text{total no. nucleotides in V region} - \text{total no. of mutations in the V region}}{\text{total no. nucleotides in V region}}$$

Any differences between the germline and expressed antibody sequences were considered to be somatic mutations unless they were at sites of junctional diversity or could be shown to arise from the PCR primers originally used to clone the antibody cDNA or if they were at known sites of allelic polymorphism.

Each somatic mutation was classed as either R or S depending on the effect on amino acid sequence. In the rare instances where there were two nucleotide substitutions in one codon, the effect of each single mutation upon the amino acid sequence was considered separately and correspondingly classed as R or S mutations.

2.15.3 Analysis of distribution of R and S mutations

Statistical analysis of the distribution of R and S mutations was carried out for all V_H and V_L sequences. The cumulative multinomial method (Lossos et al., 2000) was used to calculate the probability that an excess or scarcity of R mutations in the Ig V gene CDRs or FRs results from chance alone. Calculations by the multinomial method were made using the JAVA applet provided by Lossos *et al.*, at http://www-stat.stanford.edu/immunoglobin.

It has previously been shown from analysis of the known crystal structures of twenty-six different antigen-antibody complexes that certain residues within CDRs of the heavy and light chains are more likely than others to contact antigens (MacCallum et al., 1996). Therefore the amino acid residues most likely to contact antigen were determined according to the paper of MacCallum *et al.* (1996).

2.16 Preparation of cloned V_H and V_L sequences for expression as whole Ig

The stored cDNA of IS4 and CL24 mAbs did not contain splice sites or leader sequences, which are required to enable their secretion from mammalian cells. Thus these features had to be incorporated by PCR amplification and ligation into an intermediate (pBCVHCASS4) then final expression vectors (pLN100 for V_λ, pKN100 for V_κ and pG1D210 via pG1D106 for V_H), which also contain the appropriate constant region cDNA, necessary to allow the production of whole antibody molecules.

2.16.1 Incorporation of restriction and splice donor sites into stored cDNA by PCR amplification

Purified recombinant IS4 and CL24 V region cDNA in pGEM-T Easy vector was obtained as described in section 2.13 and then used as a substrate in a PCR reaction. The conditions for this reaction were the same as used in section 2.7, except that an annealing temperature of 42°C was used with specific primers shown in Table 2.4.

PCR amplification of cloned IS4 and CL24 V region cDNA with specific primers incorporates a *Sfi* I restriction site at the 5` end and a splice donor site followed by a *Bam*H I restriction site at the 3` end. The addition of restriction sites allows the cDNA to be ligated into subsequent vectors whilst the splice donor site at the 3` end of each V region enables a portion of DNA between the V and C regions in the final expression vector to be treated as an intron.

The primers were designed so that they overlapped with each V_H and V_L region followed by a spacer of 5-6 nucleotides then *Sfi* I and *Bam*H I restriction sites to be incorporated at the 5` and 3` ends of each V_H and V_L region respectively.

Primers	Sequences
IS4HBACK	5`GGCTC<u>GGCCACTGAGGCC</u>CACAGCCAGGTCCAGTTTGTGCAGTCT3`
IS4HFOR	5`TT<u>GGATCC</u>ACTCAC::CTGAGGAGACGATGACCAG3`
CL24HBACK	5`GGCTC<u>GGCCACTGAGGCC</u>CACAGCGAAGTGGAGTTATTGGAATCT3`
CL24HFOR	5`TT<u>GGATCC</u>ACTCAC::CCGAGGAGACGATGACCTG3`
IS4LBACK	5`GGCTC<u>GGCCACTGAGGCC</u>CACAGCCAGTCTGCCCTGACTCAGCCTG3`
IS4LFOR	5`GT<u>GGATCC</u>ACTCAC::CTAGGACGGTCAGCTT3`
CL24LBACK	5`GGCTC<u>GGCCACTGAGGCC</u>CACAGCGAAATTGTGTTGACGCAGTCT3`
CL24LFOR	5`GT<u>GGATCC</u>ACTCAC::GTCGTTTGATCTCCAGCCTGGT3`

Table 2.4 PCR primers used in eukaryotic expression system

Restriction sites are underlined

:: denotes the position of the splice donor site

The cDNA of IS4 V_H was amplified and extended by the following primers. IS4 H back primer includes a site for the enzyme *Sfi* I (GGCCNNNNNGGCC) followed by a spacer of six nucleotides and then the first seven codons of IS4 V_H. The enzyme site was bounded at the 5` end by a five bp "cap" designed to enhance its stability in the PCR product. IS4 H forward primer contains a *Bam*H I site (GGATCC) followed by a splice donor site then the 5` end of the DNA strand complementary to the last six codons of IS4V_H.

The other forward and back primers were designed to include similar elements in the same order. IS4L forward and IS4L back primers extend and amplify IS4V_L. Names of the primers for CL24 are similarly self-explanatory.

2.16.2 Digestion with *Sfi* I and *Bam*H I

The product of each PCR was confirmed by gel electrophoresis and bands of the appropriate size extracted from the gel (by method A, section 2.9) and then digested sequentially with *Sfi* I (NEB) then *Bam*H I (NEB). First a reaction mixture containing - 19μl of PCR product, 4μl 10x NEB Buffer 2, 2μl (20u/μl) *Sfi* I and ddH_20 to a final volume of 40μl was incubated at 50°C for 90 minutes. Then 5.1μl of NaCl 1M and 2μl (20u/μl) *Bam*H I were added and incubated at 37°C for a further hour. Products of the reaction were confirmed by gel electrophoresis and bands of approximately 400bp in length containing digested V region mAb cDNA were cut out and DNA extracted (by method A, section 2.9). The intermediate vector pBCVHCASS4 was digested and purified in exactly the same way.

2.16.3 Cloning into the intermediate vector pBCVHCASS4

Purified V region DNA produced by the preceding methods was then ligated into an intermediate expression vector pBCVHCASS4 described in section 2.2 and shown in Figure 2.1. Ligation of the purified product of *Sfi* I/*Bam*H I digested pBCVHCASS4 (as described above) with a PCR amplified V region DNA *Sfi* I/*Bam*H I digested fragment places the insert in frame 3` to the leader sequence ensuring it is translated in phase. These purified *Sfi* I/*Bam*H I digested PCR products were ligated with three different concentrations of *Sfi* I/*Bam*H I digested pBCVHCASS4: - neat, 1 in 5 and a 1 in 10 dilution with sterile water. To 10μl of insert/vector mix - 2μl 10x ligase buffer, 1μl (3u/μl) T4 DNA ligase and autoclaved water to 20μl were added.

The mixture was incubated at 4°C for 16-20 hours and then used to transform competent DH5α *E.coli* cells as in section 2.12. Paired controls were also set up with each concentration of vector and instead of PCR product 5μl of sterile water were added instead. An intact plasmid was also transformed as a positive control to check that the cells had been made competent. The transformed cells were spread onto LB agar plates containing 50μg/ml chloramphenicol. A sample of non-transfected bacteria was spread on a separate plate as a negative control to show that antibiotic resistance had been conferred only by transfection of plasmid. Colonies produced from an overnight incubation at 37°C were then grown overnight in 5ml LB containing 50μg/ml chloramphenicol. Glycerol stocks were made from these cultures, as in section 2.12.

Plasmid DNA was extracted from these chloramphenicol resistant clones (see section 2.13) and 4μg of DNA was digested with 2μl (10u/μl) *Hind* III and 2μl (10u/μl) *Bam*H I, 4μl 10x buffer E and ddH_2O to a final volume of 40μl. This mixture was incubated at 37°C for one hour and the products run upon a gel to check for the presence of a band at 400-450bp confirming incorporation of V region DNA into pBCVHCASS4. This band was then cut out, purified (by method A, section 2.9.1) and ligated into the final expression vectors.

2.16.4 Cloning into final expression vectors

The properties of the expression vectors pG1D106, pG1D210, pKN100, and pLN100 are described in section 2.2 and allow each V_H , V_κ or V_λ sequence cloned into pBCVHCASS4 to be ligated into a vector containing the appropriate C region DNA. In each of the final expression vectors with the exception of pG1D210, the C region DNA contains a splice acceptor site at its 5` end and a termination codon at the 3` end. The presence of the splice donor site at the 3` end of each V region introduced during the earlier PCR amplification ensures that the DNA between the V and C regions is treated as an intron by mammalian cells and spliced out of the RNA thereby producing mRNA for translation. Thus the expressed peptide will contain only the leader, V and C region sequence.

2.16.5 Transfer of heavy chain variable regions into pG1D210

After the V_H regions of both IS4 and CL24 had been successfully ligated into pG1D106 we were informed by the manufacturers of the vector (AERES Biomedical) that intronic DNA between the V_H and C_γ regions was not always spliced out by mammalian cells and could therefore be present in the whole antibody expressed from COS-7 cells. The effect of this expressed intron upon antibody structure and function was unknown. Although no effects have been seen in experiments using a variety of cloned antibodies both in our laboratory and at AERES Biomedical, a modified version of pG1D106 was created by the manufacturers. This new plasmid (pG1D210) lacked the expressed intron but still contained the same promoter, ampicillin resistance gene, origin of replication and C_γ DNA. Therefore V_H fragments of both IS4 and CL24 were transferred from pBCVHCASS4 into pG1D210.

Vector pG1D210 is designed such that the leader – V_HJ_H sequence can be ligated into it as a *Hind* III/*Xho* I fragment. The *Xho* I site occurs in the part of the MCS of pG1D210 which encodes the final few amino acids of J_H. Therefore any V_HJ_H sequence to be ligated into pG1D210 must contain a *Xho* I site at its 3` end. Alternative sites in J_H include *Kpn* I and *Bst*E II. However, due to somatic mutations in J_H, none of these sites are present in IS4V_H or CL24V_H. Therefore *Xho* I sites were introduced by PCR mutagenesis using specially designed primers shown in Table 2.5. Purified recombinant IS4 and CL24V_H region cDNA in pBCVHCASS4 was obtained as described in section 2.9 and then used as a substrate in a PCR reaction. The conditions for this reaction were the same as in section 2.7, but an annealing temperature of 55°C was used.

Primers	Sequences
IBHFOR	5`CCACTCACCG<u>CTCGAG</u>ACGATGACCAG3`
CBHFOR	5`CCACTCACCG<u>CTCGAG</u>ACGATGACCTG3`
CIHBACK	5`GAGCT<u>AAGCTT</u>GCCGCCACCA3`

Table 2.5 PCR primers used to create *Hind* III/*Xho* I fragment of IS4 and CL24 V_H
Hind III (AAGCTT) and *Xho* I (CTCGAG) restriction sites are underlined.

Specific forward PCR primers were designed to introduce a silent mutation in the nucleotide sequence CTCCTC at the 3` end of the J_H region of IS4 and CL24 in pBCVHCASS4 leading to the creation of a *Xho* I site CTCGAG, see Table 2.5. The *Xho* I site was chosen over the other possible restriction sites in the MCS of pG1D210 because it required the least change in nucleotide sequence of IS4 and CL24 J_H and could be co-digested with *Hind* III in buffer B at 37°C for one hour. The primers corresponding to the J_H region of IS4 in pBCVH4CASS and CL24 in pBCVH4CASS were named "IBH For" and "CBH For" respectively. These forward primers were used in conjunction with a back primer "CIH" that identified a sequence common to both fragments because it bound at the *Hind* III region (AAGCTT) of pBCVHCASS4, see Table 2.5.

Products of this PCR step were confirmed by gel electrophoresis (as in section 2.8), the appropriate band was purified from the gel (by method B, section 2.9.2) and then co-digested with *Hind* III and *Xho* I (instead of *Bam*H I in section 2.16.3) and cloned into pG1D210 (as in section 2.16.4).

2.16.6 Sequencing of inserts in the final expression vectors

The presence and identity of each insert in the recombinant vectors was confirmed by further sequence analysis before the DNA was transfected into eukaryotic cells. This additional step was required to ensure that no sequence errors had been introduced by PCR amplification. Sequencing and polyacrylamide gel electrophoresis were carried out as described in methods sections 2.15 and 2.15.1. Primers (shown in Table 2.6) were used which bind to the expression vectors pG1D210, pKN100 and pLN100 at distances of 30-110 base pairs from either end of the insert enabling the full length of the insert to be read.

Primers	Sequences
DONOR (binds to pLN100 80bp 3` of insert)	5`CAAAGTTCTGCCCTTGG3`
HCMV1 (binds to pG1D210, pLN100 and pKN100 50bp 5` of insert)	5`GACTAACAGACTGTTCC3`
HUCK (binds to pKN100 110bp 3` of insert)	5`CTCATCAGATGGCGGGA3`
HUG1 (binds to pG1D210 90bp 3` of insert)	5`TTGGAGGAGGGTGCCAG3`

Table 2.6 Primers used in sequencing of inserts in expression vectors

2.16.7 Site directed PCR mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene) was found to be a convenient method to correct a PCR error causing a Ser to Pro change at codon 14 of IS4V_L and produce Arg to Ser mutations at various positions in IS4V_HCDR3 to assess the effects of these mutations on binding to CL.

The basic procedure requires a double stranded DNA vector containing an insert of interest and the design of two specific oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo* DNA polymerase. This enzyme has a 6-fold higher fidelity in DNA synthesis than *Taq* DNA polymerase hence has a low potential for generating further mutations during PCR. Incorporation of the oligonucleotide primers generates a mutated plasmid. Following PCR the product is treated with *Dpn* I, an endonuclease specific for methylated DNA produced by DH5α *E.coli*, which thus digests the parental DNA template but not the mutated plasmid. Vector DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells.

The mutagenic oligonucleotide primers were designed according to manufacturers specifications. Primers were between 25 and 45 bases in length with a melting temperature (T_m) greater than or equal to 78°C. The formula used to calculate T_m is shown in Table 2.7 A) and the specific primers in Table 2.7 B).

Purified plasmid DNA was obtained as described in section 2.13. A series of PCR reactions were then set up containing 5ng, 10ng, 20, and 50ng concentrations of plasmid DNA with the appropriate mutagenic primers, see Table 2.7 B), and reaction solutions provided in the QuikChange site-directed mutagenesis kit (Stratagene). 1μl (5-50ng) of plasmid DNA was mixed with 5μl 10x reaction Buffer, 1μl of dNTP mix, 5μl forward primer (125ng), 5μl reverse primer (125ng), 1μl *Pfu Turbo* DNA Polymerase (2.5 units/μl) and autoclaved ddH_2O to a final volume of 51μl.

As a negative control no cDNA was added to one reaction mixture. Each mixture was then pulse spun and placed into a PCR machine programmed according to the manufacturers instruction with the extension time of each reaction calculated at two minutes per kb of plasmid length. Cycling parameters used were as follows: -

Initial denaturation at 95°C for 30 seconds

Then 12 cycles as follows –	Denaturation	- 95°C for 30 seconds
	Annealing	- 55°C for one minute
	Extension	- 68°C for 2 minutes per Kb for sample reactions and 12 minutes for control reaction

The reaction was then held at 4°C until the samples were removed.

Once the PCR step had been carried out the methylated template DNA containing the undesired sequence was digested by the addition of 1μl (10u) of *Dpn* I restriction enzyme directly to each amplification reaction. The resultant reaction mixture was mixed gently by pipetting up and down several times, after which it was pulse spun in a bench centrifuge and incubated at 37°C for one hour.

A) $T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$

Where - N is the primer length in bases

Values for %GC and %mismatch are whole numbers

B)

Primer	Sequence
1.	5\`CCTCCGTGTCTGGG**T**CTCCTGGACAGTCG3\`
2.	5\`CGACTGTCCAGGAG**A**CCCAGACACGGAGG3\`
3.	5\`GCGAGAGGA**A**GT**A**G**T**GACGTT**A**G**T**GGAGTTCTTTGGAGGGGCAG**T**CATGACTAC3\`
4.	5\`GTAGTCATG**A**CTGCCCCTCCAAAGAACTCC**A**C**T**AACGTC**A**C**T**AC**T**TCCTCTCGC3\`
5.	5\`CGCGAGAGGA**A**GT**A**G**T**GACGTTCGGG3\`
6.	5\`CCCGAACGTC**A**C**T**AC**T**TCCTCTCGCG3\`
7.	5\`CTGCGCGAGAGGA**A**GTCGAGACGTTCGG3\`
8.	5\`CCGAACGTCTCGAC**T**TCCTCTCGCGCAG3\`
9.	5\`CGCGAGAGGACGT**A**G**T**GACGTTCGGGGAG3\`
10.	5\`CTCCCCGAACGTC**A**C**T**ACGTCCTCTCGCG3\`
11	5\`GGACGTCGAGACGTT**A**G**T**GGAGTTCTTTGGAGG3\`
12.	5\`CCTCCAAAGAACTCC**A**C**T**AACGTCTCGACGTCC3\`
13.	5\`CTTTGGAGGGGCAG**T**CATGACTACTTTGAC3\`
14.	5\`GTCAAAGTAGTCATG**A**CTGCCCCTCCAAAG3\`

Table 2.7 Properties and sequences of primers used in site directed mutagenesis

A) Formula to calculate melting temperature of mutagenic primers.

B) Mutagenic primers with sites of point mutation shown in bold. Primers 1 and 2 were used to amplify IS4V_L and mutate a Pro to Ser residue at codon 14. Primers 3 -14 were used to amplify IS4V_H and mutate the Arg to Ser residues at the following positions; primers 3 and 4 mutated Arg 96, 97, 100 and 100g to create V_Hx; primers 5 and 6 mutated Arg 96 and 97 to create V_Hiⅈ primers 7 and 8 mutated Arg 96 to create V_Hi; primers 9 and 10 mutated Arg 97 to create $V_H$ii; primers 11 and 12 mutated Arg 100 to create $V_H$iii; primers 13 and 14 mutated Arg 100g to create $V_H$iv.

A separate 50µl aliquot of XL1-Blue supercompetent cells was then transformed with 1µl of the *Dpn* I treated DNA from each control, sample reaction and transformation control plasmid supplied by Stratagene. This method was very similar to that described in section 2.12 with the following exceptions: a heat shock of 42°C was applied for 45 seconds with recovery on ice for a further two minutes; and 0.5ml of NZY medium (see appendix D) preheated to 42°C was added to each transformation reaction.

Colonies were picked from the sample transformation plates into 5ml LB cultures containing 50µg/ml of ampicillin and kept overnight in a 37°C shaking incubator. The following day glycerol stocks were made (as in section 2.12) and plasmid DNA purified (see section 2.13) and screened for the presence of the desired mutation by sequencing (as in section 2.15).

2.16.8 Large scale extraction of DNA for transfection into COS-7 cells

The yield of plasmid DNA from a mini-prep was insufficient for electroporation therefore large-scale extraction to produce 100-500µg of DNA per preparation was carried out using the Qiagen Plasmid Maxi purification buffers (see appendix C) and protocol. A single colony of recombinant DNA in final expression vector was picked from a freshly prepared LB/Ampicillin plate to inoculate a starter culture of 5ml LB/Ampicillin medium. This culture was incubated for approximately eight hours at 37°C with vigorous shaking at approximately 300rpm. One ml of the starter culture was then diluted into 500ml of LB/Ampicillin medium and incubated at 37°C for approximately 16 hours overnight with vigorous shaking at 300rpm. Following this bacterial cells were harvested by centrifugation at 1864g for 15 minutes at 4°C. The supernatant was discarded and the bacterial pellet resuspended in 10mls of ice cold buffer P1 (for P1 - 3 see appendix C). Ten mls of buffer P2 was added. After mixing and incubation at room temperature for 5 minutes 10mls of chilled buffer P3 was added. The mixture was kept on ice for 20 minutes then centrifuged at 20,000g for 30 minutes at 4°C. Supernatant containing plasmid DNA was promptly removed and re-centrifuged at 20,000g for 15 minutes at 4°C. A Qiagen tip 500, containing a DNA binding resin, was equilibrated by the addition and free gravity flow of 10mls Buffer QBT. Supernatant was then applied to the Qiagen tip and allowed to enter and bind DNA to the resin by free gravity flow while all other components pass through. The

DNA was then washed by passing 30 ml of buffer QC through the tip twice and eluted with 15 ml of buffer QF. To precipitate the DNA 10.5 ml (0.7 volumes) of room temperature isopropanol was added to the eluted DNA, mixed and then centrifuged at 1864g for 45 minutes at 4°C. Supernatant was carefully decanted and the DNA pellet washed with 5 ml of room temperature 70% ethanol and centrifuged at 1864g for 15 minutes at 4°C. Supernatant was discarded and the pellet dried under a vacuum for 2-5 minutes, then redissolved in 1ml of autoclaved water.

The presence and concentration of purified DNA in each sample was confirmed by restriction digest (as in section 2.14) and gel electrophoresis with molecular weight markers (see section 2.8).

Recombinant DNA was then precipitated. A 2.5 times volume of 100% ice cold ethanol and 1/10 volume of 3M-sodium acetate was added and left at -70°C for 60 minutes. After this the mixture was centrifuged at 13,000g for 10 minutes at 4°C and the pellet washed with 1ml of ice-cold 70% ethanol before further centrifugation as above for 5 minutes. Precipitated DNA was then dried under a vacuum and the pellet resuspended under sterile conditions, in an appropriate volume of autoclaved water to give a final concentration of 2μg/ml and stored at -20°C.

2.17 Expression of cloned V_H and V_L sequences as whole antibodies

2.17.1 Tissue culture

All tissue culture preparation was carried out in a sterile tissue culture hood. Any buffers, solutions and materials required were kept in the tissue culture laboratory and solely used for this work. Plastic ware was supplied by Nunc, VWR International, Leicester, U.K. For growth media and solutions for maintenance of eukaryotic cell lines see appendix D.

2.17.2 Eukaryotic cell lines

COS-7 cells and CHO*dhfr*$^-$ cells were kind gifts from Alison Levy, AERES Biomedical, Mill Hill, London, U.K. The COS-7 cells were originally derived from American Type Culture Collection, Ref No. CRL 1651.

CHO*dhfr*⁻ cells used are from the cell line DXB11 originally derived from CHO-K1 in 1978 by Professor Lawrence Chasin (Professor of Biological Sciences, Columbia University, USA). These CHO-K1 cells were obtained from Ted Puck and Fa-ten Kao at the Eleanor Roosevelt Cancer Institute in Denver, Colorado, USA in 1970. One *dhfr* allele was deleted in DXB11 and the other allele carries a mis-sense mutation resulting in a single amino acid substitution (Urlaub and Chasin, 1980).

2.17.3 Transient expression in COS-7 cells

COS-7 cells are immortal monkey kidney cells, which express the T antigen of the human polyoma virus SV40 constitutively. The T antigen binds to the SV40 origin to stimulate replication. Plasmids used in this study all contain the SV40 origin (which is not otherwise present in COS-7 cells) hence will be replicated actively in COS-7 cells and achieve a high copy number. This expression system is thus ideal for achieving efficient transient expression of heavy and light chains.

2.17.4 Maintenance of COS-7 cells in tissue culture

A master cell bank of COS-7 cells frozen at as low a passage number as possible to enhance the efficiency of transfection was stored in liquid nitrogen. Aliquots of these cells were frozen in growth medium 1 (see appendix D) containing 10% DMSO which is cryoprotective during freezing but toxic to the cells once they have thawed. Thus as soon as the cell aliquot was thawed it was immediately added to 10ml prewarmed growth medium 1 and spun at 250g for 10 minutes at room temperature. Medium was then discarded and the cell pellet resuspended in 10ml growth medium 1 and transferred to an $80cm^2$ flask containing a further 10ml of the same medium.

COS-7 cells were maintained in a continuous culture by changing the growth medium every 3-4 days. All solutions and media to be used were initially placed in a 37°C water bath for 10 minutes. The $80cm^2$ or $175cm^2$ tissue culture flask containing COS-7 cells was removed from the incubator. Old medium was removed by aspiration and the remaining cells adherent to the floor of the flask washed by adding 10mls sterile HBSS to remove any remaining fetal calf serum. HBSS was then removed and 3mls of 10% Trypsin/Versene (T/V) solution added. The flask was shaken gently to allow T/V to fully cover the floor of the flask. T/V was then left in contact with the cells for two minutes in a 37°C incubator to release all cells adherent to the floor of the flask. At the end of this time the flask was tapped and viewed under the microscope to make sure at least 80% of the cells had become detached. 10mls of growth medium 1 were then added to the flask and gently shaken to inactivate the T/V solution. This mixture was then split from an 80cm^2 to a 175cm^2 flask or from one 175cm^2 to three new 175cm^{2}flasks and growth medium 1 added to a final volume of 20ml for an 80cm^2 flask or 25ml for a 175cm^2 flask. The flasks were then placed horizontally in a 37°C incubator in which the concentration of CO_2 was 5% and checked daily to assess growth.

To create a cell bank, cells of a low passage number growing in an exponential phase were trypsinised as above then spun at 250g for 10 minutes at room temperature. Following a cell count (carried out as in section 2.5.1) the cells were resuspended in the appropriate volume of growth medium 1 containing 10% DMSO, to give a concentration of 1×10^7 cells/ml. 1.5ml aliquots were then put in cryovials, immediately placed at -80°C overnight and then transferred to liquid nitrogen.

2.17.5 Transfection of recombinant expression vector into COS-7 cells

One 175cm^2 flask was prepared as described above with the exception that the final split was between two rather than three large flasks, for each V_H/V_L combination to be tested. The following day the cells in each flask were washed with HBSS and trypsinised as described above. Ten mls of warm medium 1 were added to each flask and the entire contents then transferred to a sterile 50ml centrifugation tube. The cells were then centrifuged at 250g for five minutes at room temperature. The supernatant was discarded and the pellet of COS-7 cells resuspended in 20-50 mls of autoclaved PBS according to the size of the cell pellet. A cell count was performed upon a 200μl aliquot of the suspension to estimate the number of cells present (see section 2.5.1). The remainder of the suspension was again centrifuged at 250g for 10 minutes at room temperature. Supernatant was carefully removed by aspiration and the cell pellet resuspended in the appropriate volume of PBS to give a final concentration of 1×10^7 cells per ml.

10μg of thawed recombinant vector containing the heavy chain to be tested plus 10μg of vector containing the light chain to be combined with this heavy chain were added to a disposable electroporation cuvette. 700μl of the COS-7 cell suspension was also added and the cuvette placed in a Gene Pulser II electroporator (Biorad). A pulse of charge at 1.9kV and 25μF was applied and repeated for each experimental transfection to be performed. A negative control cuvette containing COS-7 cells only and no DNA was prepared and subjected to an electroporation pulse in exactly the same way.

The COS-7 cells were allowed to recover at room temperature for 10 minutes. The contents of each cuvette were then transferred to a separate 10cm tissue culture dish containing 8ml of medium 2 (see appendix D) warmed to 37°C. Incubation of the dish in 5% CO_2 at 37°C for 72 hours then took place before harvesting the COS-7 cell supernatant for analysis. At the end of the incubation period the medium from each dish was carefully transferred by pipette into a sterile 15ml centrifuge tube. The tubes were centrifuged at 800g at room temperature for five minutes to remove cell debris. Supernatant was then removed from each tube and treated with 6u of *DNase* I after which the sample was incubated for one hour at 37°C. EDTA to a final concentration of 15mM was then added to inactivate the enzyme plus 5μl of 1M sodium azide solution. The *DNase* I was added to digest any free DNA in solution that may interfere with subsequent testing for anti-DNA activity in an ELISA as demonstrated previously (Rahman et al., 1998). Sodium azide solution prevented colonisation by microorganisms. Supernatant was stored at 4°C for testing by ELISA.

A fresh 8ml aliquot of warmed growth medium 2 was added to the COS-7 cells in each 10cm tissue culture dish and incubated at 37°C in 5% CO_2 before harvesting the COS-7 cell supernatant for analysis after a further 72 hours as explained above.

Growth medium 2 contains γ-globulin free fetal bovine serum. Thus any IgG detected in the supernatant after the 72 hour incubation is very likely to be the result of IgG production from COS-7 cells due to transfected Ig genes. An alternative post elctroporation medium 3 (see appendix D) containing a serum replacement Ultroser G (which lacks β_2GPI) instead of γ-globulin free fetal bovine serum was used when the antibodies produced were to be tested in a modified (hence β_2GPI free) CL ELISA. The negative control of COS-7 cells containing no DNA was included to confirm that no IgG production occurs without transfected IgG genes.

2.17.6 Concentration of COS-7 cell supernatant

Centricon YM-30 (Millipore) devices were used to concentrate antibody within the COS-7 cell supernatant. Concentration was performed as per the manufacturer's instructions. Two mls of supernatant was added to each sample reservoir coupled with its filtrate vial and centrifuged at 2000g at room temperature for 30 minutes. Further supernatant was then added to the sample reservoir and subsequent centrifugation steps performed for 40-50 minutes. This process was repeated until all but the final one ml of supernatant had passed through the membrane of the sample reservoir. A retentate vial was then placed over the sample reservoir the unit inverted and centrifuged at 5000g for 2 minutes to transfer concentrated antibody from the membrane into the remaining supernatant. Concentrated samples were then stored at 4°C.

2.17.7 Heat shock and Phospholipase C treatment of COS-7 cell supernatant

In some cases a comparison was made between properties of the supernatant before and after a heat shock at 56°C or digestion with Phospholipase C (PLC). After centrifuging to remove cell debris, the COS-7 cell supernatant was divided equally into two aliquots one of which was treated with PLC and the other by heat shock as performed by Cabiedes and colleagues (Cabiedes et al., 1998).

One aliquot of the supernatant was further divided in half and 10u of PLC were added to each half. The optimum conditions for PLC are incubation at 37°C in pH 7.3 for one hour. The pH of the supernatant ranged from 7.35 - 7.45 hence the incubation time was extended to two hours. In one half (called "inactivated PLC") the enzyme was inactivated immediately by adding EDTA to a final concentration of 15mM prior to incubation at 37°C for 120 minutes. In the other half (the "active PLC") the enzyme remained active during the incubation period before the EDTA was added. Hence the only difference between the two aliquots was that digestion of phospholipids occurred in one but not the other

The other aliquot of the supernatant was also divided into two halves only one of which was subjected to a heat shock at 56°C for 30 minutes. All of the supernatant treated with PLC or +/- heat shock were treated with 1M sodium azide and stored at 4°C.

2.18 Detection and characterisation of whole IgG molecules in COS-7 supernatants by ELISA

2.18.1 Detection of whole IgG molecules in COS-7 supernatants by ELISA

In all of the different ELISAs performed the 96 well plates were supplied by Nunc, VWR International, Leicester, U.K. and OD measured by the Genios plate reader (Tecan, Reading, U.K.). Each plate was marked vertically to divide it into two halves. The 36 wells at the edge of the plate were not used and henceforth all descriptions of the plate refer to the inner 60 wells only. All ELISA reagents and buffers are listed in appendix C.

Goat anti-human IgG (Fc fragment specific) was dissolved in bicarbonate (BIC) buffer to give a working solution of 400ng/ml. 50µl of this solution were added to each well of a maxisorp plate in the left (test) half of the plate. 50µl of BIC buffer alone was added to the wells in the right (control) half of the plate. Each plate was then covered and incubated overnight at 4°C. After this the plates were washed three times with PBS/0.1% Tween and 100µl of PBS containing 2% BSA were added to each well to block non-specific binding of Ig to the plastic. The plates were incubated for one hour at 37°C and then washed three times with PBS/0.1% Tween.

In order to dilute COS-7 cell supernatant in SEC buffer serially, such that for each well in the test half of the plate there was a well in the control half containing the same supernatant at the same dilution the following procedure was performed. 50µl of SEC buffer was added to each well in both halves of the plate except the top wells. 100µl of neat COS-7 cell supernatant was added to the top well and diluted down the column such that each well contained supernatant at half the concentration of the well above. Human IgG_{λ} of known concentration (100ng/ml or 10ng/ml) was loaded and diluted in the same way upon each plate to allow the construction of a standard curve relating optical density (OD) to Ig concentration. The plates were incubated for one hour at 37°C and then washed three times with PBS/0.1% Tween. Goat anti-human λ or κ alkaline phosphatase conjugate (depending on the light chain to be detected) was diluted 1 in 2500 with SEC buffer and 50µl added to each well.

After further one-hour incubation at 37°C the plates were washed three times with PBS/0.1% Tween and once with bicarbonate buffer. One tablet of p-Nitrophenyl phosphate was dissolved per 5mls of bicarbonate buffer and supplemented with 10µl of 2mM $MgCl_2$. 50 µl of this substrate solution was added to each well. The plates were then incubated at 37°C till development of a yellow colour, which took one hour. The results were read as OD at 405nm. For each dilution of supernatant the result was calculated as the difference between the OD in the test well and the control well. Thus only conjugate bound directly to IgG in that supernatant, which had been captured by goat anti-human Fc on the plate would contribute directly to the result.

2.18.2 Detection of aCL in COS-7 supernatants by standard ELISA

Wells in the test half of a polysorp ELISA plate were coated with 50µl of cardiolipin (diluted to 50µg/ml in ethanol) and 50µl of ethanol alone were added to wells in the control half. The plates were incubated uncovered overnight at 4°C. Each plate was then washed three times with PBS and 100µl of PBS containing 10% FCS (Invitrogen) were added to each well to block non-specific binding of Ig to the plastic. After incubation at 37°C for one hour plates were washed three times with PBS.

Each concentrated COS-7 supernatant to be tested was serially diluted in PBS containing 10% FCS. An identical concentration of supernatant was loaded into a well in the test half and a corresponding well in the control half of the plate. Serum containing polyclonal antibodies known to bind CL was loaded as a positive control to each plate. The total volume in each well was 50µl. Plates were then incubated for 90 minutes at 37°C after which they were washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in PBS containing 10% FCS and 50µl of this solution added to each well. After one hour at 37°C the plate was washed four times with PBS and once with BIC buffer. Addition of substrate and incubation were carried out as in section 2.18.1 with additional OD measurements taken at 30 minutes.

2.18.3 Detection of aCL antibodies in COS-7 supernatants by modified ELISA

The standard CL ELISA was modified to remove β_2GPI from the assay by replacing 10% FCS in each relevant step with 10% BSA when testing antibody combinations produced in post electroporation medium 3 (see appendix D).

2.18.4 Detection of anti-β_2GPI antibodies in COS-7 supernatants by ELISA

A maxisorp ELISA plate was coated with 50μl of β_2GPI (diluted to 10μg/ml in PBS) on wells in the test half and 50μl of PBS alone on the control half. Plates were covered and incubated overnight at 4°C. Following that each plate was washed three times with PBS. In order to block non-specific binding 50μl of 0.25% gelatin/PBS was added to each well. After incubation at 37°C for one hour plates were washed three times with PBS.

Antibody samples were serially diluted in 0.1% BSA/PBS and then loaded upon each half of the plate, as well as a positive control of serum containing polyclonal anti-β_2GPI antibodies leaving a final volume in each well of 50μl. Plates were then incubated for two hours at 37°C after which they were washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in PBS containing 0.1% BSA/PBS and 50μl of this solution added to each well. A final incubation for one hour at 37°C was performed and the plate washed four times with PBS and once with bicarbonate buffer. Addition of substrate and incubation were carried out as in section 2.18.1 with additional OD measurements taken at 30 minutes.

2.18.5 Detection of anti-dsDNA antibodies in COS-7 supernatants by ELISA

The test half of a maxisorp ELISA plate was coated with 50μl per well of purified calf thymus dsDNA (diluted to 10μg/ml in PBS) and 50μl per well of PBS alone on the control half. Purified dsDNA used in this ELISA was a kind gift from Arti Sharma. The plates were covered and incubated overnight at 4°C. Each plate was then washed three times with PBS and non-specific binding of Ig to the plastic was blocked by the addition of 150μl of PBS containing 2% casein to each well. After one hour at 37°C the blocking agent was tipped out and each plate blotted upside down on tissue paper.

Samples of concentrated COS-7 supernatant to be tested were serially diluted in PBS/0.1% Tween and identical concentrations of supernatant added to each half of the plate. A human mAb (RH-14) known to bind dsDNA was loaded as a positive control to each plate and titrated from 1:400 to 1:32400 by three fold dilutions down the column. The total volume remaining in each well was 50µl. Plates were then incubated for one hour at 37°C after which they were washed six times with PBS/0.1% Tween. 50µl of goat anti-human IgG alkaline phosphatase conjugate (diluted 1000 fold in PBS/0.1% Tween) were then added to each well. After one hour at 37°C the plate was washed six times with PBS/0.1% Tween and twice with BIC buffer. Addition of substrate and incubation were carried out as in section 2.18.1 with additional OD measurements taken at 30 minutes.

2.19 Generation of 2a2 V gene family hybrids

Once the initial V_H/V_L chain exchange experiments had been performed hybrid (CDR exchanged) light chains were created from B3V_L; UK4V_L; and IS4V_L. Hybrid light chains were composed of the sequence of one V_L up to position 106 (including FR1 and CDR1) and the sequence of another V_L from position 106 onwards (see Figure 2.9).

The hybrids were produced by digesting the recombinant pLN100 (or pLN10) expression vectors containing each V_L with the enzymes *Acc*65 I and *Pvu* I. *Acc*65 I cuts all the V_L sequences studied at position 106 and does not cut anywhere else in the vector. *Pvu* I cuts the vector at a unique site approximately 1.5Kb from the *Acc*65 I site and does not cut any of the V_L segments. Thus a *Acc*65 I/*Pvu* I digest gives two fragments of approximately 5.9Kb and 1.5Kb. The 1.5Kb fragment contains the sequence of V_L from position 106 onwards while the 5.9Kb fragment contains the rest of V_L. By ligating the 5.9Kb fragment from one V_L expression vector to the 1.5Kb fragment from another, the required hybrid V_L were made.

Two hybrid light chains one of which contained CDR1 of B3 joined with the CDR2 and 3 of UK4 (named BU) and another containing CDR 1 of UK4 and CDR 2 and 3 of B3 (called UB) had already been created by Dr Joanna Haley.

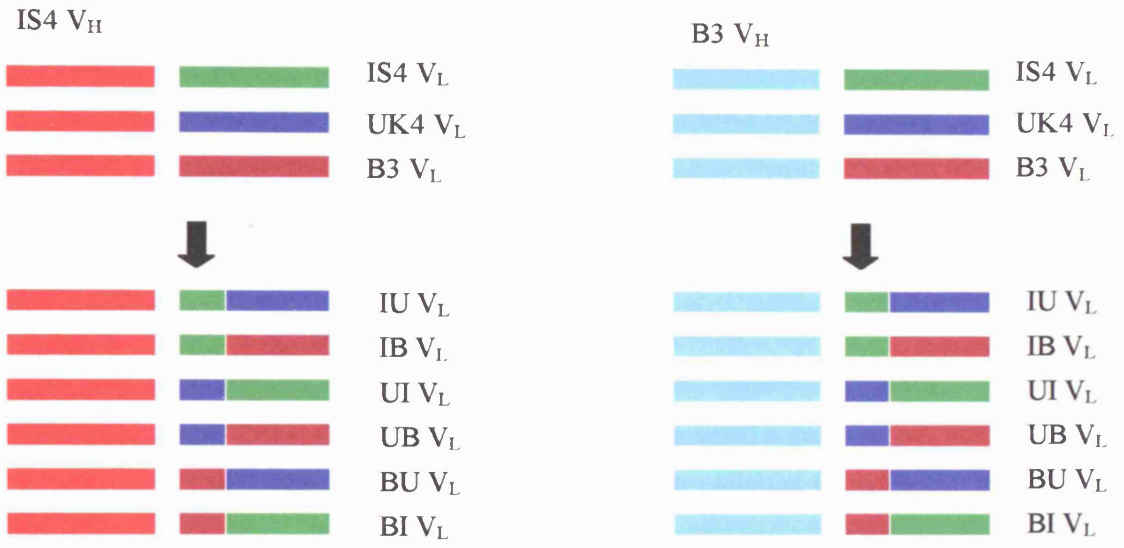


Figure 2.9 Generation of 2a2 V gene family hybrids

Schematic representation of the generation of hybrid (CDR exchanged) light chains from B3V_L; UK4V_L; and IS4V_L. Hybrid light chains were composed of the sequence of one V_L up to position 106 (including FR1 and CDR1) and the sequence of another V_L from position 106 onwards.

I created four more hybrids: - IB containing CDR1 of IS4 joined with CDR2 and 3 of B3; IU containing CDR1 of IS4 joined with CDR2 and 3 of UK4; BI containing CDR1 of B3 joined with CDR2 and 3 of IS4; and UI containing CDR1 of UK4 joined with CDR2 and 3 of UK4.

8μg of purified plasmid DNA of IS4V_L/pLN100, B3V_L/pLN10 and UK4V_L/pLN10 was obtained as described in section 2.13, and digested with 2μl (10u/μl) *Acc*65 I and 2μl (8u/μl) *Pvu* I, 10x Buffer D and ddH_20 to a final volume of 250μl. This mixture was incubated at 37°C for 18 hours and the products run upon a gel to check for the presence of two bands of appropriate size, the smaller of which contains CDR2 and 3 of each V_L plus the J_L and part of the parent plasmid and a larger band containing CDR1 of each V_L and the remaining portion of parent plasmid. The bands were then cut out, purified and ligated (as described in method B section 2.9, and section 2.10).

2.19.1 Confirmation digest of 2a2 V_L hybrids

In order to confirm the identity of V_L hybrids (IB, IU, BI and UI) generated, suitable restriction sites were chosen which would distinguish the hybrids form the "parent" plasmids (IS4V_L/pLN100, B3V_L/pLN10 and UK4V_L/pLN10) and from each other. The restriction sites which gave the most suitable fragment sizes were *Aat* II and *Ava* I. In some instances these restriction enzymes were combined with either *Hind* III or *Bam*H I to give easily distinguishable fragments. In each case 1μg of plasmid DNA was digested with either:- 5u *Aat* II in 10x buffer J; 10u *Ava* I and 10u *Hind* III in 10x buffer B; or 5u *Aat* II and 10u *Bam*H I in 10x buffer J plus ddH_20 to 20μl, as in section 2.14.

2.20 Production of Supervectors

The expression vectors pG1D210, pLN10 and pLN100 could also be used in a stable expression system. The heavy chain expression vector pG1D210 contains the *dhfr* gene whilst the light chain expression vectors pLN10 and pLN100 contain the neomycin resistance gene (neo). Therefore in CHO*dhfr*$^-$ (selective) growth medium that contains no ribonucleosides or deoxyribonucleosides but does contain neomycin, only those CHO cells with a functional *dhfr* gene and a functional neo gene would survive. Consequently the surviving cells would have the ability to produce IgG heavy chains and light chains. Although this system has been shown to work in the past, there was a risk of excess production of heavy chain compared to light chain since transfection with *dhfr* may be comparatively more productive than transfection with neo.

Therefore the two vectors were combined to produce "supervectors". From the plasmid vector pLN10 or pLN100, an *Eco*R I fragment containing the promoter, the λ constant region gene and the λ variable region gene (of the chosen antibody) was transferred into the vector pG1D210 containing IS4V_H. Three supervectors were produced: SVIS4V_H/IS4V_L; SVIS4V_H/B3V_L; and SVIS4V_H/UK4V_L. The same protocol was used to produce all of the supervectors.

2.20.1 *Eco*R I digest of IS4V_H/pG1D210 and light chain expression vector

The promoter, V_L gene and C_λ gene were all cut out of pLN10 or pLN100 by *Eco*R I restriction digest whilst pG1D210 containing IS4V_H was linearised in the same way. The restriction digests were set up with 10μg of plasmid DNA, 20u *Eco*RI, 10x buffer H and ddH_20 to a final volume of 200μl. The mixture was inverted to mix, pulsed and incubated at 37°C for 45 minutes. The presence of digested DNA was confirmed on a 0.8% agarose gel (as in section 2.8) and bands of appropriate size cut out and purified (as in method B section 2.9) for subsequent ligation.

2.20.2 Dephosphorylation of the 5` overhangs using calf intestinal alkaline phosphatase

The linearised heavy chain expression vector was treated with calf intestinal alkaline phosphatase (CIAP) which catalyses the dephosphorylation or hydrolysis of 5`-phosphate groups from DNA. Removal of the phosphate groups from both of the 5` termini of the linearised heavy chain vector reduces its ability to re-linearise and significantly increases the chance of obtaining colonies that contain the heavy chain vector and the insert (light chain DNA) following the ligation and transformation of this DNA into *E.coli.*

In a 1.5ml microfuge tube the following mixture was prepared: 200μl plasmid DNA (directly from restriction digest), 40μl 10x CIAP buffer, 1μl (1u/μl) CIAP and 159μl sterile ddH20. The contents were mixed, pulsed and incubated at 37°C for 30 minutes. The mixture was then incubated at 90°C for 10 minutes to inactivate the CIAP. The presence of digested DNA was confirmed on a 0.8% agarose gel (as in section 2.8) and bands of appropriate size cut out and purified (as in method B section 2.9) for subsequent ligation.

2.20.3 Ligation of *Eco*R I digested light chain expression vector fragment into linearised heavy chain expression vector

For each supervector, several reaction mixtures were set up containing different ratios of the *Eco*R I digested light chain vector fragment versus linearised recombinant V_H expression vector to ensure successful ligation. The amount of purified DNA of each *Eco*R I digested vector to be used was estimated by comparing the intensity of the heavy and light chain vectors upon a 0.8% agarose gel and choosing the amount of each vector which would give a 1:1 and 1:2 ratios of heavy:light chain vector. Ligation mixtures were set up containing 10-15μl of purified *Eco*R I digested heavy and light chain vector DNA in the above ratios with 10x Ligation buffer, 6u T4 DNA Ligase and sterile ddH_20 to 20μl.

Control reactions containing *Eco*R I digested heavy or light chain vector DNA only were also set up to indicate the background re-ligation of the heavy chain vector and to confirm that the light chain fragment did not contain uncut parent vector. All reaction mixtures were incubated overnight at 14°C and then transformed into competent *E.coli* cells (see section 2.12) and the colonies produced screened for the presence of supervector.

2.20.4 Large scale screening of colonies from supervector ligations

In some instances successful ligation could not be achieved after CIAP treatment of the heavy chain *Eco*R I digested fragment and this method was not always performed. Therefore large number of colonies had to be screened post ligation, because many contained re-ligated pG1D210/IS4V_H without the light chain cassette. This screening is easily achieved by the method of osmotic lysis in which 20 - 40 colonies were picked from each LB Agar plate from the above ligation and transformation. At least one control colony containing parent plasmid only was also picked. Each colony was grown in 1ml of LB broth containing Ampicillin at 37°C for 4-6 hours. A 100μl aliquot was saved from each colony and the remaining culture was pelleted in a bench top centrifuge and the supernatant removed. 50μl of Osmotic Lysis Solution (10% (w/v) sucrose, 0.25% SDS, 0.08M NaOH) were added to the pellets. The tubes were vortexed and placed at 70°C for 10 minutes then cooled on ice. 4μl of loading buffer were added and the samples loaded into wells of an agarose gel (0.8%) before the gel was submerged in buffer to prevent misloading of the viscous mixtures. Electrophoresis (15V for 16-20 hours) enabled plasmid sizes to be estimated, relative to empty vector controls. Clones of potentially the right size were selected and the remaining 100μl aliquot of culture was used to inoculate broth for plasmid purification.

2.20.5 Confirmation restriction digests of supervectors

Two confirmation digests were performed to confirm the identity of each supervector and orientation of the light chain fragment. In each case 1μg of plasmid DNA extracted from each of the supervector colonies identified by osmotic lysis was digested with either: - 10u *Hind* III, 10u *Kpn*I, 10x buffer J and ddH20 to 20μl; or 5u *Aat* II, 10x buffer J and ddH_20 to 20μl (as in section 2.14).

2.21 Stable expression system in Chinese Hamster Ovary (CHO*dhfr*$^-$) cells

2.21.1 Maintenance of CHO*dhfr*$^-$ cells prior to electroporation

The CHO cell line (DXB11) used lacks a functional *dhfr* gene. The enzyme dihydrofolate reductase is responsible for the intracellular reduction of dihydrofolic acid to tetrahydrofolic acid, which is an important co-factor in the synthesis of nucleic acid precursors. To survive the CHO*dhfr*$^-$ cell line required exogenous hypoxanthine (adenine), glycine, proline and thymidine for survival. All of these nutritional requirements were provided via non-selective medium.

If however, these CHO*dhfr*$^-$ cells were successfully transfected with expression vectors (e.g. a supervector) containing a functional *dhfr* gene the cells would be transformed to a *dhfr*$^+$ phenotype and be able to grow in selective media lacking these exogenous nutrients. Consequently CHO*dhfr*$^-$ cells were used to provide an expression system in which the genes required to produce IgG could be co-transfected with the d*hfr* gene.

The CHO*dhfr*$^-$ cells were grown in pre-warmed non-selective growth medium A (see appendix D) in 175cm^2 flasks prior to transfection and split every 3 – 4 days keeping their passage number to a minimum prior to transfection. A master cell bank was created and the cells were defrosted and frozen down as described in section 2.17.4 using growth medium A containing 10% DMSO.

To ensure exponential growth the CHO*dhfr*⁻ cells were trypsinised (with 10% T/V) 24 hours prior to transfection and divided equally between three 175cm^2 flasks containing pre-warmed growth medium A. One large flask was required for each supervector to be tested. The cells were incubated overnight at 37°C in 5% CO_2 The following day the cells in each flask were washed, trypsinised, pelleted and resuspended in sufficient autoclaved PBS to give a final concentration of 1 x 10^7 cells per ml.

2.21.2 Transfection of recombinant expression supervector into CHO*dhfr*⁻ cells

A 700μl aliquot of the washed CHO*dhfr*⁻ cell suspension was pipetted into a disposable electroporation cuvette to which 20μg of thawed recombinant supervector was added. A 1.9kV and 25μF capacitance pulse was then delivered to each experimental transfection and a control containing CHO*dhfr*⁻ cells only, using a Bio-Rad Gene Pulser® II electroporator. The cells were then allowed to recover at room temperature for 10 minutes and the contents of each cuvette transferred to a separate 10cm tissue culture dish containing 8ml of pre-warmed medium A. Each dish was incubated overnight in 5% CO_2 at 37°C.

2.21.3 Selection of transfected CHO*dhfr*⁻ cells post electroporation

From this point onwards the cells were maintained in selective growth medium B (see appendix D) which lacks ribonucleosides and deoxyribonucleosides and is absent from dialysed FCS. Thus only those cells which contain supervector and as a result a functional *dhfr* gene will then be able to grow. Following overnight growth of the transfected CHO*dhfr*⁻ cells in non-selective medium A the cells in each 10cm dish were washed with 10mls of HBSS, trypsinised and then pelleted at 250g for five minutes at room temperature. Cells from each transfection and control were resuspended separately in 100mls of prewarmed selective media B before being divided equally between ten 10cm diameter tissue culture dishes.

Incubation of the cells at 37°C in 5% CO_2 then took place for 10-14 days changing the selective medium B every 3-4 days. After 10-14 days the cells on the no DNA control dish were all dead and discrete foci of transfected cells were clearly visible on all the test culture dishes. In order to pick these foci they were visualised under a microscope and using a sterile pipette tip 200μl of foci plus media was aspirated under direct vision and transferred into individual wells of a 24 well tissue culture plate containing 0.5ml of prewarmed medium B. A total of 48 foci were picked from each transfection (ten plates). The picked cells were then grown in the selective medium B until almost confluent (between 7-14 days), changing the media every seven days.

Once the cells were almost confluent media was decanted from the individual wells of each 24 well plate and tested for the presence of whole IgG by ELISA (as described in section 2.18.1). Those clones producing the highest levels of antibody were selected for expansion in medium B until the cells were growing in $175cm^2$ flasks.

2.21.4 Assay of antibody production in transfected CHO*dhfr*⁻ cells

Following growth of the selected cells to just below confluent level in selective media in $175cm^2$ flasks the cells were washed with 10ml HBSS, trypsinised with T/V and pelleted at 250g for five minutes at room temperature. Each clone of cells was resuspended in 20ml of prewarmed selective medium B and a viable cell count performed (as in section 2.5.1). The cells were then pelleted again as above and resuspended in sufficient prewarmed medium B to produce a viable cell count of $1x10^6$ cells/ml. One ml of this solution was added to a 10cm diameter tissue culture dish containing 9ml of prewarmed medium B and incubated at 37°C in 5% CO_2 for three days. After this period, media was decanted from each 10cm dish and the concentration of intact antibody measured by ELISA (as described in section 2.18.1). Cells in each 10cm dish were washed with 5ml HBBS, trypsinised (2ml T/V) and pelleted at 250g for five minutes at room temperature. Following this the cells were resuspended in one ml of prewarmed medium B and a viable cell count was performed as above. From the new viable cell count and the antibody concentration in the decanted media the level of antibody production was calculated and expressed as ng/10^6 cells/day.

Cell banks of the clones with the highest antibody production rates were created by suspending 1 x 10^7 cells/ml in medium B containing 10% DMSO and stored in liquid nitrogen (as in section 2.17.4) for further selection.

CHAPTER 3

RESULTS OF SEQUENCE ANALYSIS OF IS4 AND CL24 ANTIBODIES

3.1 Amplification of V_H and V_L cDNA by PCR

Initially I was provided with hybridomas secreting mAb of unknown sequence by Dr Chen. Therefore I had to sequence and analyse these mAb myself prior to subsequent expression experiments. The V_L sequences of IS4V_L and CL24V_L were successfully amplified as shown in Figure 3.1. IS4V_λ belongs to the V_λ 2 family as demonstrated in Figure 3.1 A). A band of 330 base pairs in length corresponding to IS4 V_λ was amplified by HL20 and JL primers only. In the same way CL24V_κ was found to belong to the V_κ III family, see Figure 3.1 B). I was unable to amplify IS4V_H and CL24V_H using the VH9 primer. However, I amplified these V_H sequences successfully using the VH1 and VH3 primers designed using sequences provided by Dr Chen once his group had completed their own sequence analysis of IS4 and CL24. The results of V_H sequence amplification are shown in Figure 3.2.

3.2 Small scale extraction of cloned cDNA in pGEM-T Easy Vector

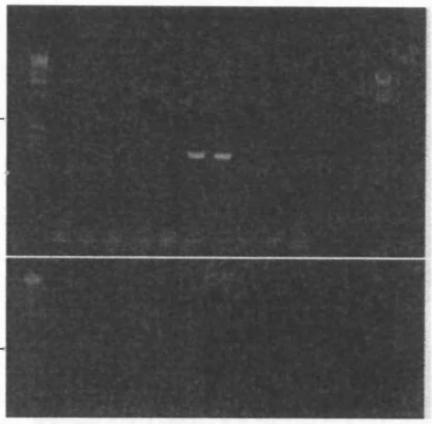
Successful ligation of each V_H and V_L chain into pGEM-T Easy Vector and subsequent cloning into *E.coli* was confirmed by an *Eco*R I digest of recombinant DNA extracted from overnight cultures of white colonies grown upon ampicillin plates. A successful ligation, transformation and digestion yields a 350-400bp insert as well as a band containing pGEM-T Easy Vector of 3399bp upon gel electrophoresis shown in Figure 3.3.

3.3 Sequence analysis of IS4 and CL24 in pGEM-T Easy Vector

In Figure 3.4 a representative sequencing gel of IS4V_H and CL24V_H cloned into pGEM-T Easy Vector is shown reading through CDR2 of each heavy chain. Sequence characteristics of IS4 and CL24 V_H and V_L regions are displayed in Table 3.1. The sequences of all four V regions are shown in Figures 3.5 – 3.8 and display the positions of the CDRs and FRs identified by the Kabat system (Wu and Kabat, 1970) and contact sites as defined by MacCallum *et al.*(1996). Most of these contact sites are within the CDRs but it is important to note that not all CDR residues are contact sites and that some FR residues can be contact sites.

506, 517bp

506, 517bp



13 14 15 16 17

Top lanes in A)
1 & 12 = 1 Kb marker
2 - 5 = IS4 cDNA & HL10 primers
6 = no cDNA & HL10 primers
7 & 8 = IS4 cDNA & HL20 primers
9 - 11 = no cDNA & HL20 primers

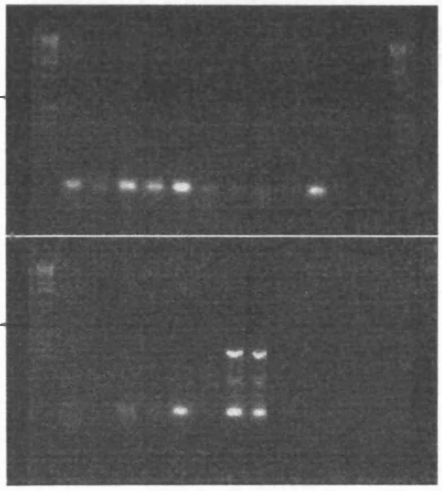
Bottom lanes in A)
13 = 1 Kb marker
14-16 = IS4 cDNA & HL30 primers
17 = no DNA & HL30 primers

B)

1 2 3 4 5 6 7 8 9 10 11 12

506, 517bp

506, 517bp



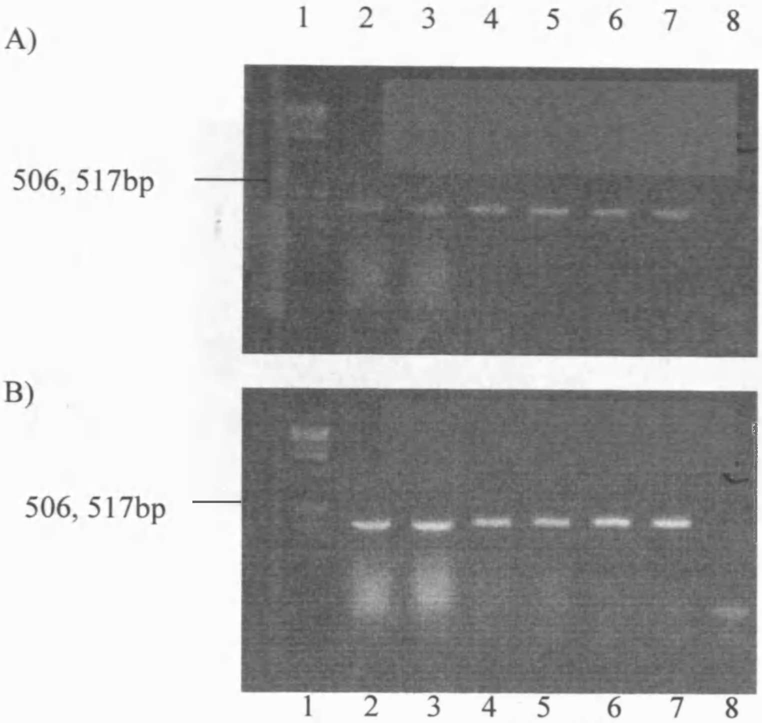
13 - - - - 18 20 21

Top lanes in B)
1 & 12 = 1 Kb marker
2 - 5 = cDNA & VK1 primers
6 = no cDNA & VK1 primers
7 - 10 = cDNA & VK2 primers
11 = no DNA & VK2 primers

Bottom lanes in B)
13 = 1 Kb marker
14 -18 = no cDNA & VK3 primers
20-21 = cDNA & VK3 primers

Figure 3.1 PCR amplification of IS4 and CL24 light chain cDNA

A) IS4 cDNA and V_λ1-3 family light chain PCR primers. A 330bp band of cDNA consistent with IS4V_L is seen only in lanes 7 and 8 with the HL20 (V_λ2 family) primers. B) CL24 cDNA and V_κ1-3 family light chain PCR primers. A 324bp band of cDNA consistent with CL24V_L is seen only in lanes 21 and 22 with the VK3 (V_κ3 family) primers.

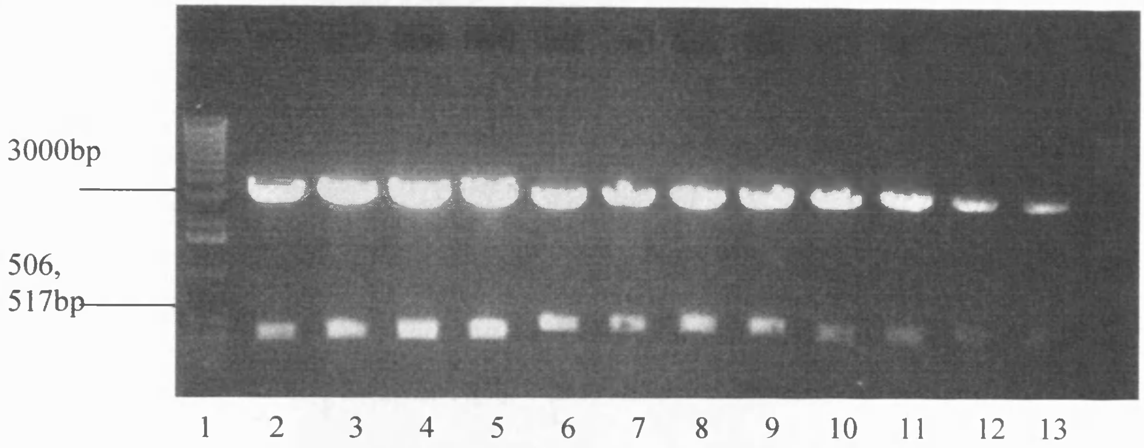


Lanes in A)
1 = 1 Kb marker
2-7 = IS4 cDNA & VH1 primers
8 = no cDNA & VH1 primers

Lanes in B)
1 = 1 Kb marker
2-7 =CL24 cDNA & VH3 primers
8 = no DNA & VH3 primers

Figure 3.2 PCR amplification of IS4 and CL24 heavy chain cDNA

A) IS4 cDNA and primers specifically designed to amplify IS4V_H. Bands of cDNA 400bp in size consistent with IS4V_H are seen in lanes 2 – 7 with the VH1 (V_H1 family) primers. B) CL24 cDNA and primers specifically designed to amplify CL24V_H. Bands of cDNA 389bp in size consistent with CL24V_H are seen in lanes 2 – 7 VH3 (V_H3 family) primers.



Lanes
1 = 1Kb marker
2 – 5 = *Eco*R I digested IS4V_L/pGEM-T Easy Vector
6 – 9 = *Eco*R I digested IS4V_H/pGEM-T Easy Vector
10 – 11 = *Eco*R I digested CL24V_L/pGEM-T Easy Vector
12 – 13 = *Eco*R I digested CL24V_H/pGEM-T Easy Vector

Figure 3.3 *Eco*R I digest of IS4 and CL24 heavy and light chain variable regions in pGEM-T Easy vector

In lanes 2 – 13 the larger fragments are identical and contain the 3399bp *Eco*R I digested pGEM-T Easy Vector. The smaller band contain variable region DNA of: - 336bp for IS4V_L in lanes 2 – 5; 390bp for IS4V_H in lanes 6 – 9; 330bp for CL24V_L in lanes 10 – 11; and 369bp for CL24V_H in lanes 12 – 13.

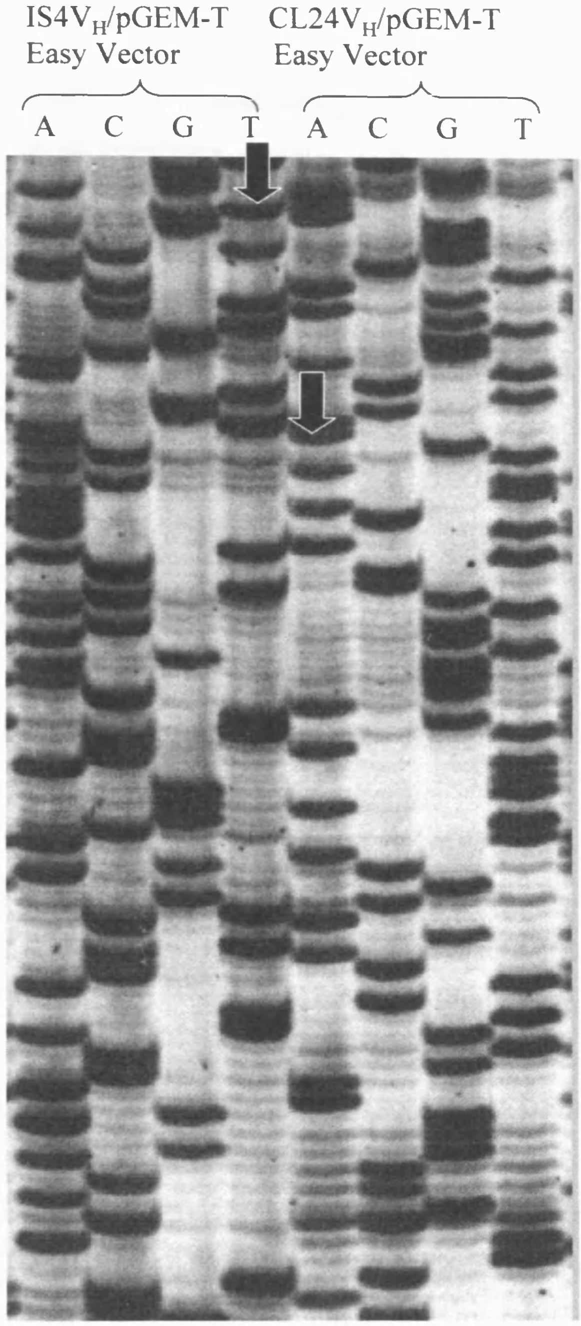


Figure 3.4 Sequence gel of IS4 and CL24 heavy chain variable regions cloned into pGEM-T Easy Vector

This figure shows samples of the sequences of IS4V$_H$ and CL24V$_H$ as read from manual sequencing gels. Sequences can be read downward from the top of the gel, from position 145 to 225 for IS4V$_H$ (compare Fig 3.6) and position 123 to 203 for CL24V$_H$ (compare Fig 3.8). The beginning of CDR2 is shown with an arrow in each sequence.

The sequences of $IS4V_L$ and $CL24V_L$ and sequence analysis of IS4 and CL24 V_H and V_L regions shown in this thesis constitute original work. The V_H sequences of these mAb however, were derived using primers designed based upon sequences provided by Dr Chens group.

3.4 Sequence analysis of IS4

3.4.1 The light chain

The V region of $IS4V_L$ shares 93.3% nucleotide and 83.4% amino acid homology with the 2a2 germline gene, Figure 3.5. The sequence of the first 26 nucleotides is determined by the PCR primer rather than the sequence of cDNA itself. Therefore any differences between the germline gene and $IS4V_L$ in this region could not be considered true somatic mutations. Hence the initial 26 nucleotides encoded by the PCR primer HL20 were not included in the calculations of nucleotide homology or significance of distribution of R/S mutations.

The 2a2 gene contains no known sites of allelic polymorphism (VBASE) hence any differences between the two sequences are likely to be due to somatic mutation. Within $IS4V_L$ there are a total of 20 nucleotide changes compared to 2a2. In the CDRs there are 12 R mutations (five of which occur in CDR3) and only two S mutations. The FRs contain six R mutations and no S mutations. This distribution of R/S mutations is suggestive of antigen drive and confirmed by the significant Pm values (see Table 3.1) of 0.0389 in the FRs (where a lack of R mutations is significant) and 0.0006 in the CDRs (indicating a significant clustering of R mutations). Interestingly in CDR3, a serine (Ser) residue is replaced by an Asn although this is not a contact residue. There are no other somatic mutations to Arg, Asn or Lys in any contact region and no overall change in charge of the CDRs.

The J region aligns with J_L3b the final 24 nucleotides of which are determined by the J_L PCR primer.

3.4.2 The heavy chain

The closest alignment of $IS4V_H$ is with the V1-03 germline gene as shown in Figure 3.6. Homology is 92.5% at the nucleotide level and 86.7% at the amino acid level.

Antibody	Family	V Gene	Nucleotide homology	V_D	V_J		No. R	No. S	Pm
IS4 V_L	$V_{\lambda}2$	2a2	93.3%	-	JL3b	FR	6	0	0.0389
						CDR	12	2	0.0006
IS4 V_H	V_H1	V1-03	92.5%	NM	JH4b	FR	10	4	0.1187
						CDR	6	2	0.1441
CL24 V_L	$V_{\kappa}III$	A27	90.9%	-	JK2	FR	8	6	0.0291
						CDR	10	0	0.0206
CL24 V_H	V_H3	V3-23	84%	D6-19	JH4b	FR	15	18	0.0195
						CDR	8	6	0.2554

Table 3.1 Sequence Features of IS4 and CL24

Abbreviations:- NM – No Match; FR – Framework Region; CDR – Complementarity Determining Region; R – Replacement mutation; S – Silent mutation; No. – number of R or S mutations in CDR/FR; Pm – p value from multinomial method of Lossos *et al.* (2000) that represents the probability that the observed distribution of R and S mutations could have arisen by chance alone i.e. without antigen driven selection.

```
     FR1                                                                        CDR1
     Q   S   A   L   T   Q   P   A   S   V   S   G   S   P   G   Q   S   I   T   I   S   C   T   G   T>A
2a2  CAG TCT GCC CTG ACT CAG CCT GCC TCC GTG TCT GGG TCT CCT GGA CAG TCG ATC ACC ATC TCC TGC ACT GGA ACC
IS4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- G--

                                                     FR2
     S   S   D   V>I G   G>S Y   N   Y>S V   S   W   Y   Q   Q>H H>L P   G   K   A   P   K   L   M>I I
2a2  AGC AGT GAC GTT GGT GGT TAT AAC TAT GTC TCC TGG TAC CAA CAG CAC CCA GGC AAA GCC CCC AAA CTC ATG ATT
IS4  --- --- --- A-- --- TC- --- --- -C- --- --- --- --- --- --C -T- --- --- --- --- --- --- --- --A ---

         CDR2                    FR3
     Y   E>D V   S   N>S R   P   S   G   V   S   N   R   F   S   G   S   K   S   G   N>F T   A   S   L
2a2  TAT GAG GTC AGT AAT CGG CCC TCA GGG GTT TCT AAT CGC TTC TCT GGC TCC AAG TCT GGC AAC ACG GCC TCC CTG
IS4  --- --C --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- TT- --- --- --- ---

                                                                 CDR3
     T   I   S   G   L   Q   A>P E   D   E   A   D   Y   Y   C   S>C S   Y   T   S>T S>I S>N T   L>W
2a2  ACC ATC TCT GGG CTC CAG GCT GAG GAC GAG GCT GAT TAT TAC TGC AGC TCA TAT ACA AGC AGC AGC ACT CTG
IS4  --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- T-- --- --- --- -CT -T- -A- --- TG-

     V   F   G   G   G   T   K   L   T   V   L   G
JL3b GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
IS4  --- --- --- --- --- --- --- --- --- --- --- ---
```

Figure 3.5 Sequence of IS4V$_\lambda$ compared to 2a2 and J$_L$3b germline genes

The positions of CDRs and FRs are as defined by Wu and Kabat (1970). Regions encoded by PCR primers are underlined and contact residues as predicted by MacCallum et al. (1996) are highlighted.

The first 21 nucleotides of the V region are encoded by a PCR primer specifically designed to amplify IS4V_H, hence this region was included in the homology calculations. None of the differences observed between IS4V_H and V1-03 occur at a site of known polymorphism. Fourteen nucleotide differences occur between the two sequences in the FRs leading to 10 R mutations and four S mutations. Six R mutations and only two S mutations occur in CDR 1 and 2. The distribution of somatic mutation found is not very suggestive of antigen driven selection and the non significant Pm values for both the FR and CDR confirm this, see Table 3.1. These calculations however, do not include CDR3 which could not be matched to a germline gene and also contributes to the antigen binding site. IS4V_HCDR3 contains five Arg residues which may have arisen as a result of antigen drive.

Again there was no overall effect upon charge by the somatic mutations. The H2 contact region however contains three Asn and one Lys residue which are all germline encoded and does not contain any acidic residues. In the H3 contact region there are four Arg and two acidic residues.

No match could be made for the D region. The reason for this is that the DNAPLOT software used very stringent criteria for aligning Ab sequences to germline D segments, described in section 1.7.7.2. The J region aligns with J_H4b.

3.5 Sequence analysis of CL24

3.5.1 The light chain

In CL24V_κ, shown in Figure 3.7, the initial 23 nucleotides are encoded by the VK3 primer used in PCR amplification. Therefore the first 23 nucleotides were not included in calculations of homology. Hence CL24 V_κ shares 90.9% nucleotide and 84.1% amino acid homology with the germline gene A27. There are no known sites of allelic polymorphism in A27 thus all differences seen between the two sequences can be ascribed to somatic mutation. In the CDRs there are ten R mutations (of which seven occur in CDR1) and no S mutations at all. This pattern is very suggestive of antigen drive with a Pm of 0.0206. The FR has eight R mutations and six S mutations also with a significant Pm of 0.0291.

	FR1																								
	Q	V	Q	L>F	V	Q	S	G	A	E	V	K	K	P	G	A	S	V	K	V	S	C	K	A	S>F
V1-03	CAG	GTC	CAG	CTT	GTG	CAG	TCT	GGG	GCT	GAG	GTG	AAG	AAG	CCT	GGG	GCC	TCA	GTG	AAG	GTT	TCC	TGC	AAG	GCT	TCT
IS4	---	---	---	T--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--A	---	---	---	---	---	-T-

						CDR1					FR2													
	G	Y	T>S	F	T	S	Y	A>F	M>L	H	W	V	R	Q	A	P	G	Q	R>G	L>P	E	W	M	G
V1-03	GGA	TAC	ACC	TTC	ACT	AGC	TAT	GCT	ATG	CAT	TGG	GTG	CGC	CAG	GCC	CCC	GGA	CAA	AGG	CTT	GAG	TGG	ATG	GGA
IS4	---	---	-G-	---	---	--T	---	TT-	T-A	---	---	---	---	---	---	---	---	---	G--	-C-	---	---	---	---

	CDR2																	FR3							
	W	I	N	A>S	G	N	G	N	T	K	Y	S	Q	K>N	F	Q	G	R	V	T>S	I	T	R	D	T
V1-03	TGG	ATC	AAC	TCG	GGC	AAT	GGT	AAC	ACA	AAA	TAT	TCA	CAG	AAG	TTC	CAG	GGC	AGA	GTC	ACC	ATT	ACC	AGG	GAC	ACA
IS4	---	---	---	T--	---	---	---	---	---	---	--C	---	---	--C	---	---	---	---	---	T--	---	---	--A	---	---

	S	A	S	T>V	A>V	Y	M	E	L	S	S	L	R>G	S	E	D	T	A	V	Y	Y	C	A	R
V1-03	TCC	GCG	AGC	ACA	GCC	TAC	ATG	GAG	CTG	AGC	AGC	CTG	AGA	TCT	GAA	GAC	ACG	GCT	GTG	TAT	TAC	TGT	GCG	AGA
IS4	---	---	---	GT-	-T-	---	---	---	---	--T	---	---	G--	---	---	---	---	---	---	---	---	--C	---	---

	CDR3														
	G	R	R	D	V	R	G	V	L	W	R	G	R	H	D
IS4	**GGA**	**CGT**	**CGA**	**GAC**	**GTT**	**CGG**	**GGA**	**GTT**	**CTT**	**TGG**	**AGG**	**GGC**	AGA	CAT	GAC

	Y	F	D	Y>F	W	G	Q	G	T	L	V	T>I	V	S	S
J_H4b	TAC	TTT	GAC	TAC	TGG	GGC	CAG	GGA	ACC	CTG	GTC	ACC	GTC	TCC	TCA
IS4	---	---	---	-T-	---	---	---	---	---	---	---	-T-	---	---	---

Figure 3.6 Sequence of IS4 V_H compared to V1-03 and J_H4b

For footnote see figure 3.5.

An increase in the number of basic residues is found in CDR1 with a serine replaced by an Arg in codon 28 and in codon 31 which is a site of antigen contact. A Tyrosine is mutated to Asn in codon 32. Overall, however, the net charge is unchanged because of two mutations where a glycine is replaced with Asp at codon 50 in CDR2 and a Ser mutated to another Asp at codon 93 in CDR3 which are both sites of antigen contact.

The J region aligns to the $J_{\kappa}2$ gene with one S mutation and one R mutation of a Lys to Arg.

3.5.2 The heavy chain

Figure 3.8 demonstrates the sequence of CL24V_H to have 84% nucleotide and 76.5% amino acid homology with V3-23 germline gene. The 47 nucleotide differences found do not occur at sites of known allelic polymorphism and are not due to the PCR primers used. CDRs 1 and 2 contain eight R mutations and six S mutations a pattern that is not suggestive of antigen drive with a non significant Pm value of 0.2554. The FRs contain 15 R mutations and 18 S mutations and have a significant Pm of 0.0195. Somatic mutations lead to the formation of basic residues at sites of antigen contact in H2 and H3 and no acidic residues in any of the CDRs.

The D region could be matched to D6-19 germline gene and the nucleotide differences give rise to four R and three S mutations in this region. J_H4b encodes the J_H region with four nucleotide differences leading to two R mutations and two S mutations.

3.6 Modelling of three-dimensional structures

To ascertain whether arginines residues hypothesised to be important in binding to CL are in fact surface exposed and hence available to bind antigen, modeling studies were carried out by Dr Sylvia Nagl, shown in Figure 3.9. The three-dimensional structures of CL24V_L and IS4V_L were modeled using SWISS-MODEL (Guex and Peitsch, 1997) and the coordinates of B3V_L as a template (Kalsi et al., 1996; Rahman et al., 2001). IS4V_H was modeled using B3 and 1VGE.pdb (chain H) as templates. Molecular graphics were generated in WebLab Viewer.

```
     FR1                                                                          CDR1
     E   I   V   L   T   Q   S   P   G   T   L   S>Y L   S   P   G   E>D R   A   T   L   S   C   R   A
A27  GAA ATT GTG TTG ACG CAG TCT CCA GGC ACC CTG TCT TTG TCT CCA GGG GAA AGA GCC ACC CTC TCC TGC AGG GCC
CL24 --- --- --- --- --- --- --- --- --- --- --- -A- C-- --- --- --- --T --- --- --- --- --G --- --- ---

                                         FR2
     S   Q   S>R V   S>G S>R S>T Y>N L>V A   W   Y   Q   Q   K   P>F G   Q   A   P   R   L   L   I   Y
A27  AGT CAG AGT GTT AGC AGC AGC TAC TTA GCC TGG TAC CAG CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT
CL24 --- --- --A --- G-- --G -CG A-- G-- --- --- --T --- --- --G TT- --- --- --- --- --- --- --- --- ---

     CDR2                        FR3
     G>D A   S   S   R   A   T   G   I   P   D   R   F   S   G   S   G   S   G   T   D   F   T   L   T>I
A27  GGT GCA TCC AGC AGG GCC ACT GGC ATC CCA GAC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT CTC ACC
CL24 -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T-

                                                         CDR3
     I   S   R   L>M E   P   E   D   F   A   V>I Y   Y   C   Q   Q   Y   G   S>D S   P
A27  ATC AGC AGA CTG GAG CCT GAA GAT TTT GCA GTG TAT TAC TGT CAG CAG TAT GGT AGC TCA CCG
CL24 --- --- --- A-- --- --- --- --C --- --- A-A --- --- --C --- --- --- --- GA- --- ---

     Y   T   F   G   Q   G   T   K>R L   E   I   K
Jκ2  TAC ACT TTT GGC CAG GGG ACC AAG CTG GAG ATC AAA
CL24 --- --C --- --- --- --- --- -G- --- --- --- ---
```

Figure 3.7 Sequence of CL24 V_L compared to A27 and J_K2

For footnote see figure 3.5.

FR1

E V Q>E L L E S G G G L V Q P G G S L R L S C A A S
V3-23 GAG GTG CAG CTC TTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT
CL24 --A --- G-- T-A --- --A --- --- --- --- --- --G --- --G --- --- --- --- --- --- --- --- --- --- ---

CDR1 **FR2** **CDR2**

G F T>S F>L S>A S Y>H A M S W V R Q A>R P G K G L E W V>I S A>S
V3-23 GGA TTC ACC TTT AGC AGC TAT GCC ATG AGC TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTC TCA GCT
CL24 --- --- T-- --A GC- --- C-- --- --- --- --- --- --T --A CG- --C --A --- --- --A --- --- A-- --- AG-

FR3

I S>T G>I S>R G G S T>I Y Y A D S V K G R F T I S R D N S
V3-23 ATT AGT GGT AGT GGT GGT AGC ACA TAC TAC GCA GAC TCC GTG AAG GGC CGG TTC ACC ATC TCC AGA GAC AAT TCC
CL24 --- -C- ATC C-- --- --G --T ATT --T --- --- --- --T --- --- --- --C --- --- --- --- --- --- --C ---

K>R N T>I L Y L Q>E M N S>N L R A E D T A V>L Y Y>H C A K>R
V3-23 AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCC GTA TAT TAC TGT GCG AAA
CL24 -G- --T -TC --A --- --- G-- --- --- -A- T-- --G --- --- --- --- --- T-- --- C-- --- --- -G-

CDR3

R G I>S A V A>I G>R S
D6-19 AGG GGT ATA GCA GTG GCT GGT ACC
CL24 --- --C -GT --- --- AT- A-A T--

Y>H F D Y W G Q G T L>Q V T>I V S S
J_H4b TAC TTT GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA
CL24 C-- --- --- --- --- --- --- --- --- -A- --- -T- --- --- --G

Figure 3.8 Sequence of CL24 V_H compared to V3-23, D6-19 and J_H4b
For footnote see figure 3.5.

The models show that multiple surface exposed Arg residues are prominent features of IS4V_H and CL24V_L. CL24V_L has two surface exposed Arg residues (positions 28 and 31) in CDR1, which have both been produced as a result of somatic mutation, see Figure 3.9 A). There are germline encoded Arg residues at position 54 in CDR2 and position 61 in FR3, see Figure 3.9 A). In contrast, IS4V_L has only one surface exposed germline encoded Arg residue, at position 54, see Figure 3.9 B). In the Arg rich CDR3 of IS4$V_{H,}$ four out of five Arg residues (at positions 96, 97, 100 and 100g) are predicted by the model to be surface exposed and therefore potentially important in binding to CL, shown in Figures 3.9 C) and D).

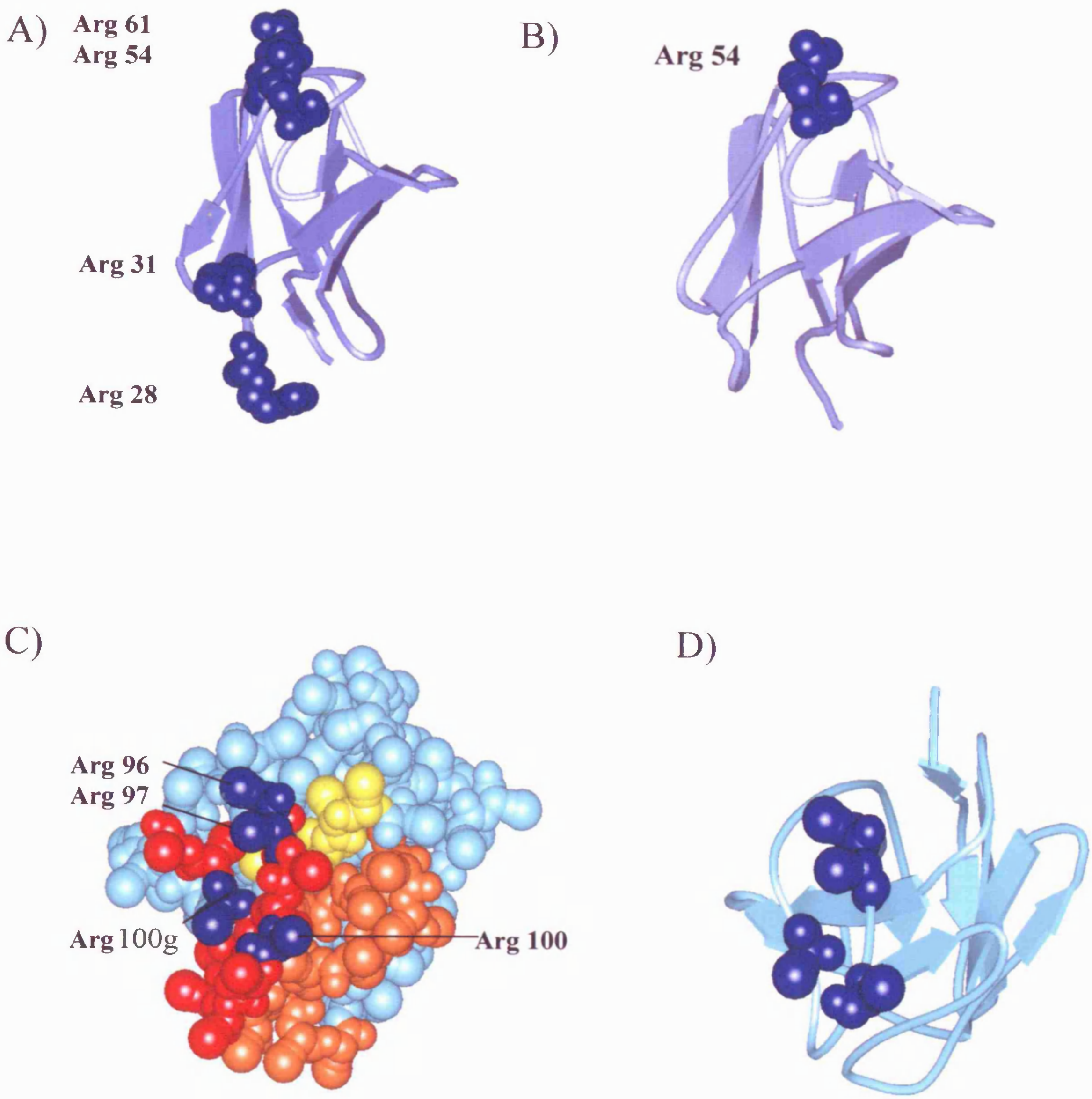


Figure 3.9 Modelling studies of CL24V$_L$, IS4VL and IS4V$_H$

A) Model of the light chain of CL24 is shown in *secondary structure mode*. B) A model of the light chain of IS4 in *secondary structure mode*. C) Spacefill model of the heavy chain of IS4. The labelled arginines (Arg) in the large CDR3 loop region of IS4V$_H$ are predicted to importantly contribute to CL-binding specificity. D) The heavy chain of IS4 in *secondary structure mode*. In all models Arg residues predicted to be exposed are shown in *dark blue* (*spacefill mode*); CDR1 in light and heavy chains is depicted in *yellow*, CDR2 in *orange* and CDR3 in *red*.

3.7 Discussion of results

When studying monoclonal antibodies such as IS4 and CL24 derived from different patients, uncertainty arises as to whether such antibodies are truly representative of pathogenic antibodies found in other patients with APS. Thus it is important to consider to what extent the sequences of these two antibodies are in agreement with what has been previously discovered about human aPL. In particular: - the pattern of gene usage; degree of somatic mutation; evidence of antigen drive; and existence of any sequence motifs likely to be important in binding to CL/β_2GPI must all be considered in IS4 and CL24.

3.7.1 Pattern of gene usage

Previous analyses have shown that the aPL most likely to be representative of those developing in patients with APS are those in which the V_H and V_L sequence are encoded by commonly rearranged genes (Giles et al., 2003a). The light chains of IS4 and CL24 both utilise some of the most commonly expressed V_L genes, 2a2 and A27 respectively, (Cox *et al.*, 1994; Ignatovich *et al.*, 1997; Brezinschek *et al.*, 1998). The J_L gene usage of both antibodies J_L3b for IS4 and J_κ2 for CL24 conforms to the known usage of these genes in peripheral lymphocytes where J_L2 or J_L 3 and J_κ2, J_κ3 and J_κ4 genes are most commonly expressed (Dorner *et al.*, 1999; de Wildt *et al.*, 2000).

CL24V_H utilises one of the most commonly rearranged genes V3-23 (Brezinschek et al., 1997; de Wildt et al., 2000). IS4V_H, however, is encoded by the germline gene V1-03, which is not amongst those genes most commonly expressed in healthy individuals and patients with SLE (Cox *et al.*, 1994; Brezinschek *et al.*, 1997; Ignatovich *et al.*, 1997; de Wildt *et al.*, 2000). Furthermore, in a systematic analysis of all published aPL sequences, V1-03 was not found to encode any of 36 published human IgM or IgG monoclonal aPL (Giles et al., 2003b).

IS4V_HCDR3 could not be matched to any known D_H gene through VBASE and this may be because this region of the antibody is in fact highly mutated hence it cannot be matched to any known germline genes. The D segment of CL24V_H uses the D6 family; D6-19 gene thus differing from the most commonly expressed D2 and D3 families (Brezinschek et al., 1997; Corbett et al., 1997; de Wildt et al., 2000). Both aPL however use the J_H4b gene which is one of the most commonly rearranged J_H genes in peripheral B cells (de Wildt et al., 2000).

Therefore the pattern of gene usage in CL24 and IS4V_L is that which would be expected from the intrinsic bias of the recombination mechanism. Genes that are commonly rearranged by that mechanism are also commonly used to encode both IgM and IgG aPL. The gene usage of IS4V_H however, is unusual and it seems unlikely that the germline sequence of this gene is predisposed to formation of a CL binding site. Therefore unless the entire CL binding ability of IS4 is conferred by the light chain there must another distinguishing feature of this aPL heavy chain, such as somatic mutations and/or certain sequence motifs in V_HCDR3.

3.7.2 Degree of somatic mutation

The V regions of both antibodies demonstrate a high degree of somatic mutation. The greatest numbers of somatic mutations were found in CL24 particularly in the heavy chain and a similar pattern was found in IS4. IS4V_H is highly mutated with only 92.5% homology to the germline gene. However, many of these mutations are R in FRs or S mutations in CDRs, with the result that statistical analysis of the pattern of mutations does not suggest that they have been selected by antigen. Although this analysis cannot exclude the possibility that an individual mutation in IS4V_H CDR1 or 2 could be playing a role in binding to CL, there are only four R mutations in these CDRs of which only one is a mutation to Asn with no mutations to Arg or Lys.

3.7.3 Evidence of antigen drive

Statistical analysis of CL24 using the multinomial method of Lossos *et al.* (2000) reached levels of significance in both the heavy and light chain sequence. In IS4 only the V_L sequence reached levels of significance. The multinomial analysis only examines the V_H region up to the end of FR3 and does not include CDR3 of each heavy chain. Therefore although a non significant p value was obtained for IS4V_H, the CDR3 of this heavy chain may have arisen as a result of antigen drive. CDR3 has been shown to be important in conferring binding of anti-dsDNA antibodies to dsDNA in previous studies (Li et al., 2000) and the same may well hold true for pathogenic aPL which share some similarities with the former antibodies (Rahman et al., 1996).

3.7.4 Evidence of any sequence motifs likely to be important in binding to CL/β_2GPI

Overall the heavy chain CDRs of both IS4 and CL24 display an accumulation of predominantly Arg and Asn residues compared to acidic residues greater than that seen in the light chains. The numerous Arg residues found in IS4V_HCDR3, which far outnumber any acidic residues, may also be conferring a strong affinity for antigen (Figure 3.6). CL24V_H demonstrates a greater number of somatic mutations in all CDRs leading to the formation of Arg residues in CDR2 and 3 (Figure 3.8).

IS4V_HCDR3 is long (15 amino acids) with a very high content of five Arg residues. It seems likely that the length and Arg content of this CDR3 arise from N addition and somatic mutation and that selection by antigen has acted to promote expansion of the clone containing these Arg residues. Models in Figures 3.9 C) and D) support this hypothesis because four of the five Arg residues in CDR3 are surface exposed and could therefore be important in contributing to CL-binding specificity.

The model of CL24V_L in Figure 3.9 A) clearly shows that the two Arg residues created by somatic mutation in CDR1 (at position 28 and 31) are surface exposed. In contrast, IS4V_L shown in Figure 3.9 B) has no somatic mutations to Arg at all but does contain a germline encoded Arg at position 54 which is also present in CL24V_L.

3.7.5 Conclusion

In conclusion IS4 and CL24 are highly mutated human IgG aPL. Both chains of CL24 and IS4V_L utilise commonly expressed V_H and V_L genes and are thus representative of other pathogenic aPL. The heavy chain of IS4V_H, however, does not utilise a commonly expressed gene but does contain a long Arg rich CDR3 which could not be matched to germline gene. On the whole the heavy chains carry more somatic mutations than the light chains, but the distribution of R and S mutations in the latter is more suggestive of antigen driven clonal expansion as measured by a statistical method. A greater number of Arg, Asn or Lys residues some of which are somatically mutated, are found in the CDRs of the heavy chains compared to the light chains. Both somatic mutations and Arg residues are likely to be important in determining the ability of IS4 and CL24 to bind PL/β_2GPI. Hence the purpose of subsequent experiments was to examine which features of which chains were the most important.

CHAPTER 4

RELATIVE IMPORTANCE OF DIFFERENT HUMAN aPL DERIVED HEAVY AND LIGHT CHAINS IN THE BINDING OF aPL TO CARDIOLIPIN

4.1 PCR amplification of IS4 and CL24

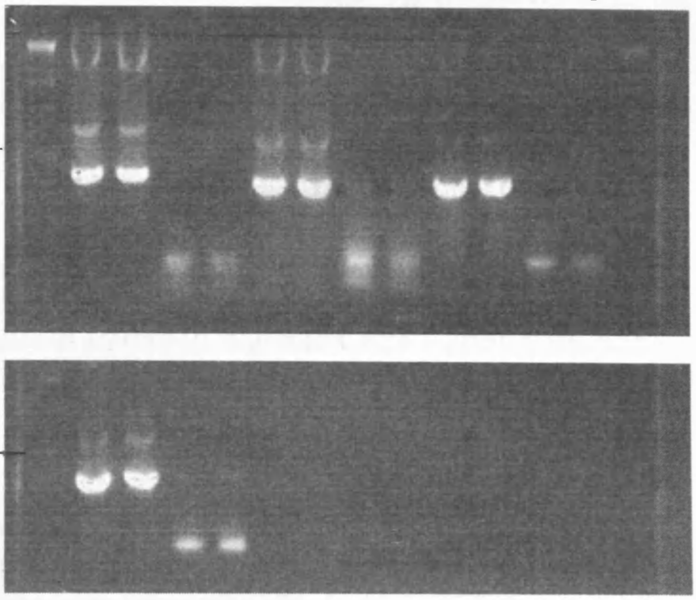
The first step in this process was to PCR amplify cloned V region DNA of IS4 and CL24 with specific primers to add a *Sfi* I restriction site at the 5` end and a splice donor site followed by a *Bam*H I restriction site at the 3` end. Successful PCR products were confirmed by gel electrophoresis. Figure 4.1 demonstrates a band of approximately 350 base pairs for each V_L region and approximately 400 base pairs for each V_H region compared to a negative control in which no DNA was added to the PCR mixture to confirm that non-specific amplification did not occur.

4.2 Cloning into the intermediate vector pBCVHCASS4

Purified IS4 and CL24 PCR products from the previous step were then *Sfi* I/*Bam*H I digested and ligated into pBCVHCASS4, which had been digested by the same enzymes. Successful ligation was confirmed by dual digestion with *Hind* III/*Bam*H I. Figure 4.2 shows an agarose gel of *Hind* III/*Bam*H I digested recombinant plasmid DNA in which the upper band of DNA contains pBCVHCASS4 3446bp in size and the lower bands show inserts of the appropriate size in A) of 463bp for IS4 V_H and 442bp for CL24 V_H and in B) of 406bp for both IS4 V_L and CL24 V_L.

4.3 Cloning into the final expression vectors

Hind III/*Bam*H I digested insert was purified and ligated into pLN100, pKN100 for the light chains and pG1D106 for the both heavy chains. The presence of insert was confirmed by a *Hind* III/*Bam*HI digest of the recombinant plasmids. In Figure 4.3 A) lanes 2 – 11 two separate bands of DNA are visible, the larger containing pG1D106 (7328bp) and the smaller CL24V_H (442bp). A similar pattern is seen in Figure 4.3 B), with lanes 2 – 6 containing pLN100 (7451bp) and IS4 V_L (406bp) and lanes 7 – 8 containing pKN100 (7664bp) and CL24 V_L (406bp). IS4V_H was not successfully cloned into pG1D106.



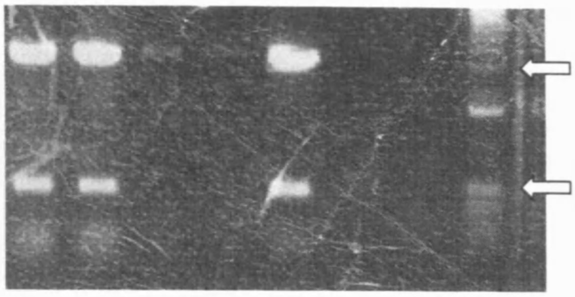
Lanes in A)
1 = 1Kb marker
2 – 3 = IS4 cDNA PCR amplified with IS4HF and HB primers
4 – 5 = no cDNA PCR amplified with IS4HF and HB primers
6 – 7 = IS4 cDNA PCR amplified with IS4LF and LB primers
8 – 9 = no cDNA PCR amplified with IS4LF and LB primers
10–11 = CL24 cDNA PCR amplified with CL24HF and HB primers
12–13 = no cDNA PCR amplified with CL24HF and HB primers

Lanes in B)
1 = 1Kb marker
2 – 3 = CL24 cDNA PCR amplified with CL24LF and LB primers
4 – 5 = no cDNA PCR amplified with CL24LF and LB primers

Figure 4.1 PCR amplification of IS4 and CL24 V region cDNA to incorporate *Sfi* I, *Bam*H I restriction sites and a splice donor site

A) The following PCR products are visible; - 410bp DNA fragment containing IS4V_H (in lanes 2 and 3); a 354bp DNA fragment containing IS4V_L (in lanes 6 and 7); and a 389bp DNA fragment containing CL24V_H (in lanes 10 and 11). A control reaction with each set of PCR primers and no cDNA did not yield a product. B) A 353bp DNA fragment containing CL24V_L is present in lanes 2 and 3 with no product when the same PCR reaction and primers were used with no cDNA.

A)



1 2 3 4

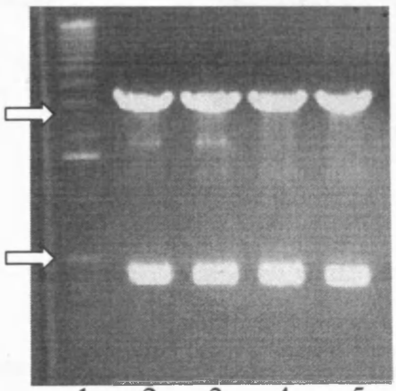
Lanes in A)

1-2 = *Bam*H I/*Hind* III digested IS4V_H/pBCVHCASS4

3 = *Bam*H I/*Hind* III digested CL24V_H/pBCVHCASS4

4 = 1 Kb marker, 3054bp (upper) and 506, 517bp (lower) bands arrowed

B)



1 2 3 4 5

Lanes in B)

1 = 1 Kb marker, 3054bp (upper) and 506, 517bp (lower) bands arrowed

2 & 3 = *Bam*H I/*Hind* III digested IS4V_L/pBCVHCASS4

4 & 5 = *Bam*H I/*Hind* III digested CL24V_L/pBCVHCASS4

Figure 4.2 *Bam*H I/*Hind* III digest of IS4 and CL24 heavy and light chains in pBCVHCASS4

A) A 463bp fragment of DNA is seen in lanes 1 and 2 containing IS4V_H and a 442bp fragment in lane 3 containing CL24V_H, the larger fragments in lanes 1 – 3 are 3446bp long containing the remainder of pBCVHCASS4.

B) The *Bam*H I/*Hind* III digested fragments of DNA are: - 406bp containing IS4V_L in lanes 2 and 3; and 406bp containing CL24 V_L in lanes 4 and 5. The larger fragments in lanes 2 – 5 are 3446bp long containing the remainder of pBCVHCASS4.

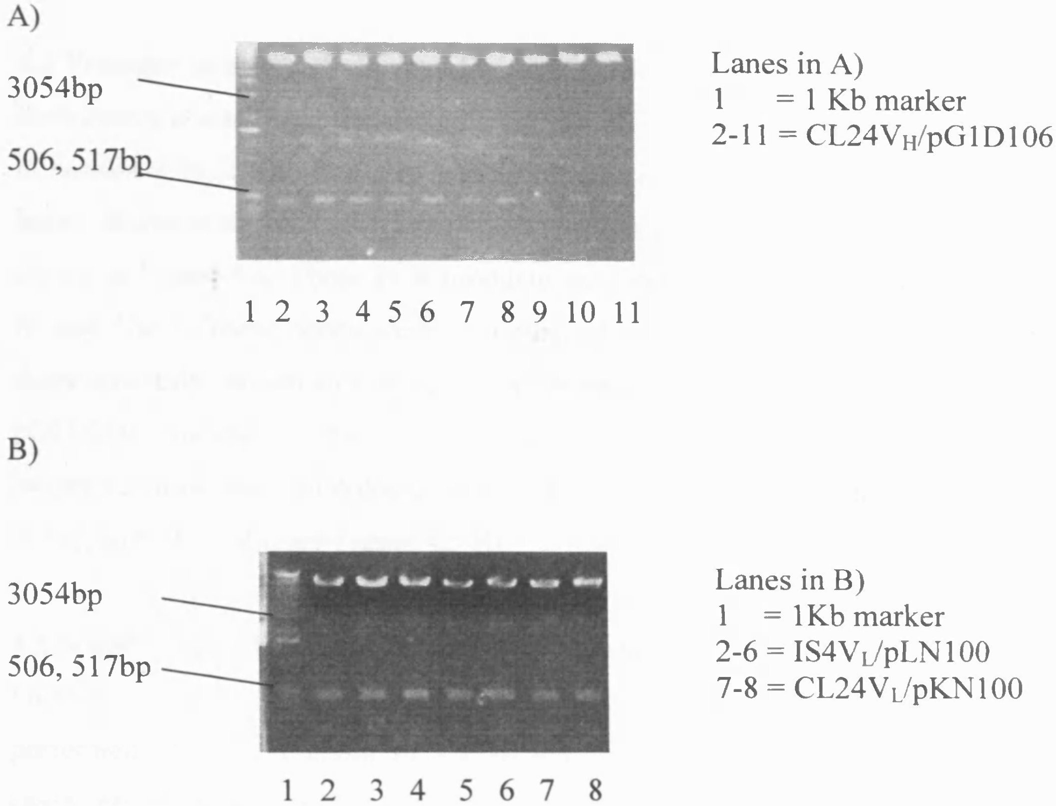


Figure 4.3 Cloning into expression vectors pG1D106, pLN100 and pKN100

In the above figures each lane represents plasmid derived from a different colony on LBAgar plates. A) *Hind* III/*Bam*H I digest of CL24 V_H/pG1D106 in lanes 2 – 11 reveals a larger band of DNA containing pG1D106 (7328bp) and the smaller CL24V_H (442bp). B) A *Hind* III/*Bam*H I digest of: - IS4V_L/pLN100 (lanes 2 – 6) reveals two bands of DNA one containing pLN100 (7451bp) and the other IS4 V_L (406bp); and CL24V_L/pKN100 in lanes 7 – 8 gives a large band of DNA containing pKN100 (7664bp) and a smaller CL24 V_L (406bp) band.

4.4 Transfer of heavy chain variable regions into pG1D210

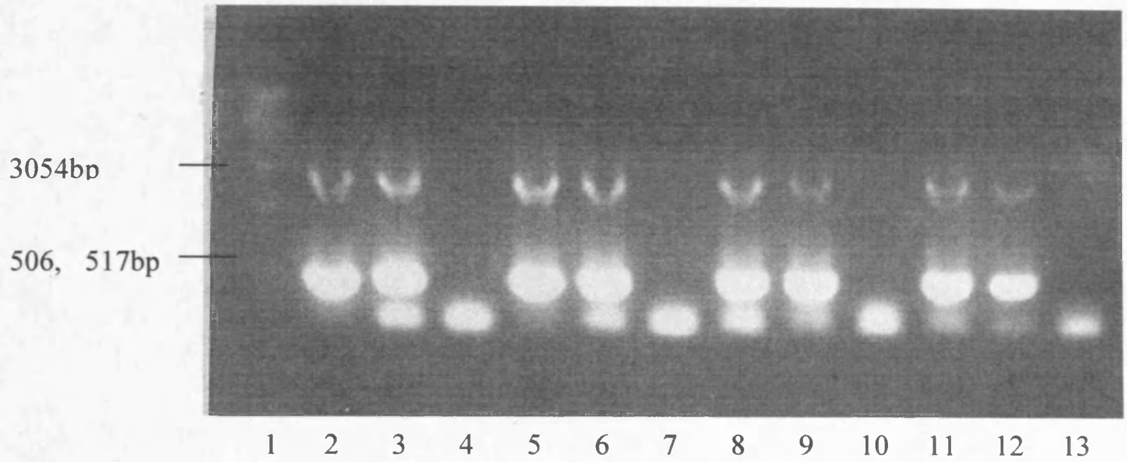
Both heavy chains were transferred from pBCVHCASS4 into pG1D210 for reasons given in section 2.16.5. The first step was to introduce a *Xho* I site into the J_H region of both heavy chains at codon 111 – 113, which was achieved by PCR, the products of which are shown in Figure 4.4. These PCR products were then purified and dual digested with *Hind* III and *Xho* I. These bands were then purified and the amount of DNA quantified by gel electrophoresis, shown in Figure 4.5 A) before cloning different insert:vector ratios into pG1D210. Success of the ligation was determined by screening recombinant DNA recovered from bacterial colonies post ligation by *Hind* III/*Xho* I digest for the presence of IS4V_H and CL24 V_H, see Figure 4.5 B).

4.5 Sequencing of inserts in the final expression vectors

Further sequence analysis of each insert in the appropriate recombinant vectors was performed. The light chain of CL24 and both heavy chains were identical to those characterised in section 3.4 and 3.5. An example of a sequencing gel containing both IS4V_H and CL24V_H that have been PCR amplified and ligated into pG1D210 is shown in Figure 4.6 and the beginning of CDR2 highlighted. A PCR error however, had occurred in IS4V_L leading to a replacement mutation in FR1 of Ser to Pro at codon 14. To rectify this PCR error, site directed mutagenesis of IS4V_L was performed as described in section 2.16.7 with the primers shown in Table 2.7 B). Comparative sequencing gels of IS4V_L before and after the mutagenesis are shown in Figure 4.7 A) and B) respectively.

4.6 Expression of whole IgG antibodies in COS-7 supernatants

To study the relative contribution of sequence features in the heavy and light chains of IS4 and CL24 to their ability to bind CL, each heavy chain was expressed with a range of different light chains. Conversely, each light chain was expressed with a range of different heavy chains. A total of four heavy chains and five light chains were studied the sequence characteristics of which are summarized in Table 4.1. The patients from whom these mAbs were isolated have previously been described in section 2.4.

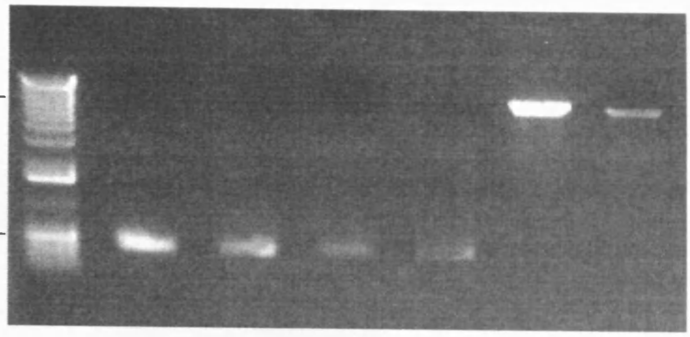


Lanes	1	= 1Kb marker
	2-3, 5-6	= CL24V_H with CHB primers
	4 and 7	= No DNA with CHB primers
	8-9, 11-12	= IS4V_H with IHB primers
	10 and 13	= No DNA with IHB primers

Figure 4.4 Introduction of *Xho* I restriction site into IS4V_H and CL24V_H

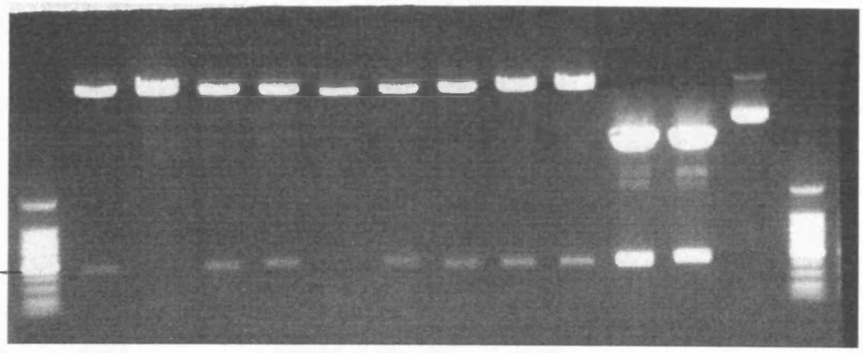
PCR amplification of CL24V_H (442bp in lanes 2, 3, 5 and 6) and IS4V_H (463bp in lanes 8, 9, 11 and 12).

A)



Lanes in A)	1	= 1Kb marker
	2 – 3	= Purified *Hind* III/*Xho* I digested CL24V_H
	4 – 5	= Purified *Hind* III/*Xho* I digested IS4V_H
	6 – 7	= Purified *Hind* III/*Xho* I digested pG1D210

B)



Lanes in B)	1 and 14	= 100bp ladder
	2 – 4 & 8 – 10	= *Hind* III/*Xho* I digested CL24V_H/pG1D210
	5 – 7	= *Hind* III/*Xho* I digested IS4V_H/pG1D210
	11	= *Hind* III/*Xho* I digested CL24V_H/pGEM-T Easy vector
	12	= *Hind* III/*Xho* I digested IS4V_H/pGEM-T Easy vector
	13	= Uncut pG1D210

Figure 4.5 Ligation of *Hind* III/*Xho* I digested heavy chains into pG1D210

A) Confirmation gel of purified *Hind* III/*Xho* I digested CL24V_H (442bp in lanes 2 -3), IS4V_H (463bp in lanes 4 – 5) and pG1D210 (7222bp in lanes 6 -7) DNA used in subsequent ligations. B) Screening colonies (lanes 1 – 10) post ligation of *Hind* III/*Xho* I digested IS4V_H and CL24V_H into *Hind* III/*Xho* I digested pG1D210. Heavy chain inserts of CL24V_H (442bp) are visible in lanes 2, 4, 8, 9 and 10 whilst IS4V_H (463bp) is visible in lanes 5 and 7. Recombinant pGEM-T Easy vector containing CL24V_H and IS4V_H was *Hind* III/*Xho* I digested and run for comparison in lanes 11 and 12.

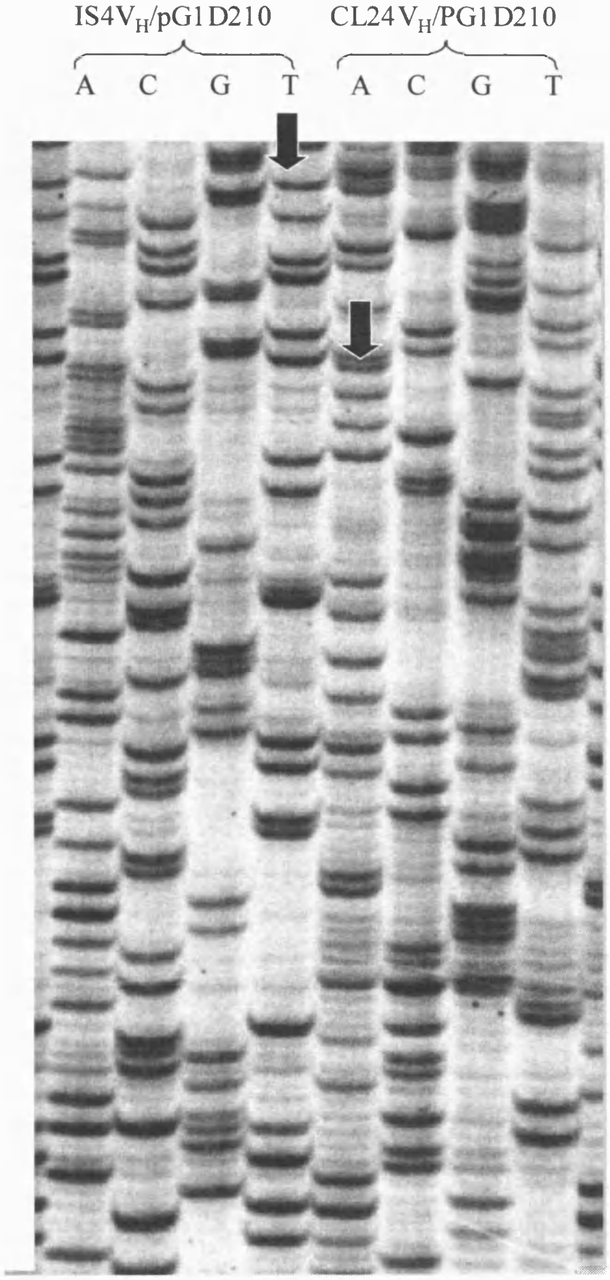
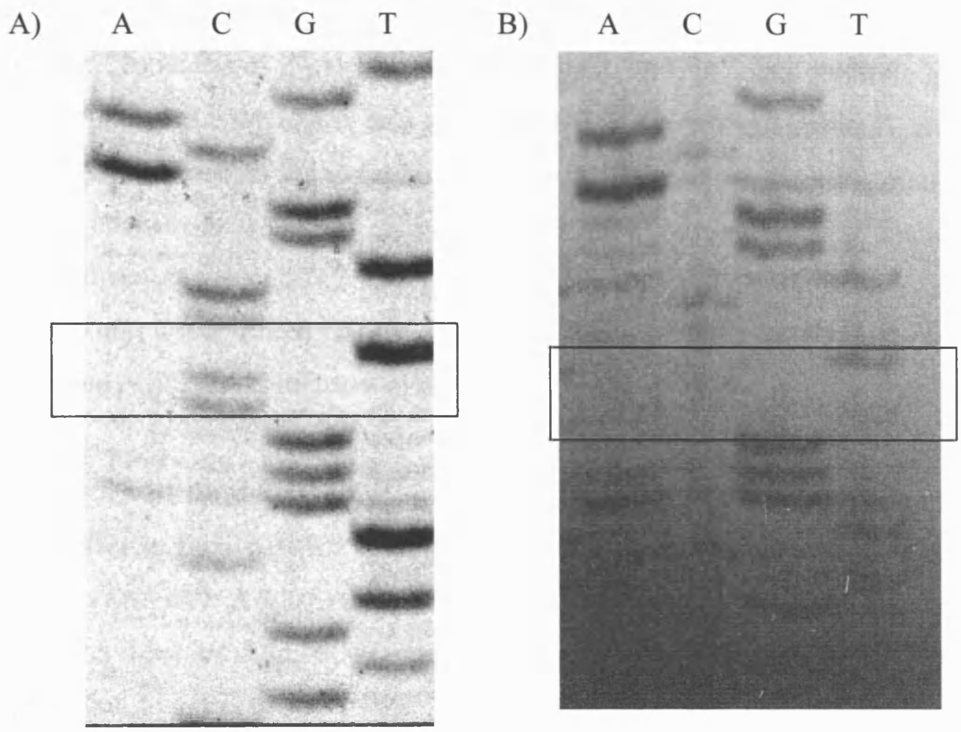


Figure 4.6 Sequence gel of IS4 and CL24 heavy chain variable regions cloned into pG1D210

This figure shows samples of the sequences of IS4V_H and CL24V_H as read from manual sequencing gels. Sequences can be read downward from the top of the gel, from position 145 to 237 for IS4V_H (compare Fig 3.6) and position 123 to 216 for CL24V_H (compare Fig 3.8). The beginning of CDR2 is shown with an arrow in each sequence.



C) **FR1**

	Codon 10									20
Germline 2a2	GTG	TCT	GGG	TCT	CCT	GGA	CAG	TCG	ATC	ACC
Sequencing gel A)	---	---	---	C--	---	---	---	---	---	---
Sequencing gel B)	---	---	---	T--	---	---	---	---	---	---

Figure 4.7 Sequence gels of IS4V$_L$/pLN100 before and after site directed mutagenesis

Manual sequencing gels of IS4VL/pLN100 containing a PCR error before, shown in A) and after site directed mutagenesis, shown in B). Sequences can be read upwards from the bottom of the gel, from position 28 to 49. A) The *Taq* polymerase induced serine to proline replacement mutation at codon 14 is boxed off. B) The same region of IS4V$_L$ is shown post site directed mutagenesis to revert the proline back to the germline encoded serine residue (shown in the box). C) The relevant sequence of IS4V$_L$ is shown for comparison.

4.7 Sequences of light chains expressed

Amino acid sequences of IS4V_L, UK4V_L, B3V_L and 33H11V_L and germline gene 2a2, are shown in Figure 4.8 A). CL24V_L and germline gene A27 are displayed in Figure 4.8 B). All of these light chains with the exception of 33H11V_L contain numerous somatic mutations. Previous statistical analysis has shown that the observed pattern of R mutations in the CDRs of these sequences (except 33H11) is consistent with antigen driven selection (Winkler *et al.*, 1992; Ehrenstein *et al.*, 1994; Menon *et al.*, 1997; Rahman *et al.*, 1998; Chukwuocha *et al.*, 2002; Rahman *et al.*, 2002).

The V_L sequences of IS4, B3, 33H11 and UK-4 are all encoded by the germline V_λ gene 2a2, but differ in their patterns of somatic mutation. In each of these light chains, some mutations create new Arg or Asn residues; see Figure 4.8 A). In CDR3 of IS4V_L a Ser residue is replaced by Asn. B3V_L contains adjacent Arg residues in CDR1, which have been produced by somatic mutations. The CDR3 of 33H11V_L contains two Arg residues, one of which is created by somatic mutation whilst the other is formed by the junction between 2a2 and $J_\lambda 2$. UK4 has a single somatic mutation to Arg in CDR3. In CDR1 of CL24V_L there are two Arg residues created by somatic mutation.

4.8 Sequences of heavy chains expressed

The amino acid sequences of each V_H chain and corresponding germline gene are displayed in Figure 4.8 C). The heavy chains all contain numerous somatic mutations. CL24 CDR2 and CDR3 each contain a codon somatically mutated to Arg. B3V_H has a single somatic mutation to Arg in CDR2. The CDR2 of IS4 V_H contains an Asn residue created by somatic mutation. Statistical analysis has shown that the pattern of R mutations in these antibodies is suggestive of antigen driven selection, with the exception of IS4V_H. However IS4V_H contains five Arg residues in CDR3 as described in section 3.4.2.

mAb	Origin	V_H Gene	Nucleotide Homology (%)	V_L Family/Gene	Nucleotide Homology (%)	Binding
CL24	SAPS	V3-23	84	V_κIII/A27	90.9	β_2GPI /CL
IS4	PAPS	V1-03	92.5	V_λ2/2a2	93.3	β_2GPI /CL
UK4	SLE	V3-74	95	V_λ2/2a2	94	CL
B3	SLE	V3-23	94	V_λ2/2a2	93	dsDNA, CL
33H11	SLE	V3-07	95	V_λ2/2a2	99.4	dsDNA

Table 4.1 Sequence characteristics of monoclonal antibodies expressed in COS-7 cell supernatant

IS4 & CL24 kindly donated by Dr P. Chen (University of California at Los Angeles), UK4 - produced by Dr S. Menon (University College London), B3 - produced by Dr M. Ehrenstein (University College London), 33H11 - produced by Dr T. Winkler, Erlangen, Germany.

A) Lambda chains

```
       FR1                      CDR1           FR2             CDR2    FR3                              CDR3     Jλ
       1                  20    24 27       34 35  40          50        60        70        80         89          100
       |                  |     |  | abc     | |    |          |         |         |         |          |           |
2a2    QSALTQPASVSG.SPGQSITISC TGTSSDVGGYNYVS WYQQHPGKAPKLMIY EVSNRPS GVSNRFSGSKSGNTASLTISGLQAEDEADYYC SSYTSSST VVFGGGTKLTVLG
IS4    ------------.---------- --A---I-S--S-- ---HL-------I-- D--S--- ------------F----------P-------- C---TIN- W------------
UK4    ------------.---------- ---SN---S----- ---L---E----L-- DAIK--- ---------E-----------------G---- ----NR.. -F-----------
B3     ------------.---------- ---RR------F-- --------------- ---H--- ---T---A----S------------------- ---S-TTR -------------
33.H11 ------------.---------- ------I------- ----------L---- D--T--- -------------------------------- ---R---- R------------
```

B) Kappa chains

```
       FR1                     CDR1           FR2             CDR2    FR3                              CDR3    Jκ
                                     31
                                     | a
A27    EIVLTQSPGTLSLSPGERATLSC RASQSVSSS..YLA WYQQKPGQAPRLLIY GASSRAT GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC QQYGSSP YTFGQGTKLEIK
CL24   -----------Y----D------ ----R-GRT..NV- -----F--------- D------ -----------------I---M------I--- ----D-- -------R----
```

C) Heavy chains

```
       FR1                           CDR1  FR2            CDR2              FR3                              CDR3            JH
                                   30 31    36  40        50           60          70            82        90         100      101
                                    | |     |   |         |            |           |             | abc      |          |        |
1-03   QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYAMH WVRQAPGQRLEWMG WINSGNGNTKYSQKFQG RVTITRDTSASTAYMELSSLRSEDTAVYYCAR                 YFDYWGQGTLVTVSS
IS4    ---F--------------------F--S-- --FL- --------GP---- -------------N--- --S--------VV-------G----------- GRRDVRGVLWRGRHD ---F-------I---

3-23   EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVS AISGSGGSTYYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK                 YFDYWGQGTLVTVSS
CL24   --E------------------------SLA -H--- ----R-------I- S-TIR---I-------- ---------R-I---E--N-------L-H--R RGSAVIRS        H--------Q-I---
B3     ----V------------------T-----T T---- -------------- T---R-S-----G--Q- ---L------S--------------------- PNVGSGW         S--S-----L-T---

3-07   EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYWMS WVRQAPGKGLEWVA NIKQDGSEKYYVDSVKG RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR                 YFDYWGQGTLVTVSS
33.H11 ---------------------------I-- NF--T -------------- --N-G--KRS------- --------V----------------------- LV              -C-------------
```

Figure 4.8 Sequence alignments of expressed V_L and V_H regions

Sequences have been aligned using DNAplot VBASE (Tomlinson et al., 1996). A) Sequences of expressed Vλ regions compared to 2a2. B) Sequences of expressed Vκ regions compared to A27. C) Sequences of expressed V_H regions compared to 1-03 (IS4), 3-23 (CL24 and B3) and 3-07 (33H11). Only the D_H region of CL24 could be matched to a germline gene (D6-19) using DNAplot and somatic mutations in this region are underlined.

The amino acids are numbered according to Kabat. Dots have been inserted to facilitate the alignment. A dash indicates homology with the corresponding germline sequence.

Abbreviations:- FR – Framework region; CDR – Complementarity determining region.

4.9 Detection of whole IgG molecules in COS-7 supernatants by ELISA

Each of the four heavy chains was paired with each of the five light chains so that a total of 20 heavy/light chain combinations were expressed in COS-7 cells. At least two expression experiments were carried out for each combination. IgG was obtained in the supernatant for 19 of the 20 combinations. The exception was 33H11V_H/CL24V_L, from which no IgG was obtained despite repeated attempts. It is possible that the structure of this heavy chain is such that it cannot form a stable antibody with a κ chain, or that the patterns of somatic mutations in these two chains lead to three-dimensional structures that are sterically incompatible.

The concentration of IgG in each COS-7 cell supernatant is shown in Table 4.2. In each case the negative control sample in which COS-7 cells were electroporated without any plasmid DNA contained no detectable IgG. Consistently high yields were obtained with the B3V_H/IS4V_L and B3V_H/CL24V_L combinations compared to the other antibody combinations. The phenomenon of variable expression with different V_H and V_L constructs is well documented both in this antibody expression system and others (Rahman *et al.*, 1998; Rahman *et al.*, 2001) although the reason for its occurrence is not clear.

4.10 Results of aCL ELISA

For each heavy/light chain combination which bound CL, the linear portion of the binding curve for absorbance against antibody concentration was determined empirically by dilution of antibody over a wide range of concentrations. Similar patterns of binding were obtained with each antibody combination from repeated expression experiments hence representative results from two separate experiments are shown in Figure 4.9 A) and B).

Only five combinations showed any binding to CL. These were IS4V_H/B3V_L, IS4V_H/CL24V_L, IS4V_H/IS4V_L, IS4V_H/33H11V_L and B3V_H/B3V_L. Despite being tested over a wide range of antibody concentrations, none of the other combinations showed any binding to CL at all.

Heavy chain	Light chain	IgG Concentration (ng/ml)	CL Binding	DNA Binding
CL24	CL24	69.5 - 310	-	-
CL24	B3	260 - 475	-	-
CL24	UK4	88 - 360	-	-
CL24	IS4	430 - 1190	-	-
CL24	33H11	32 - 33.5	-	-
B3	B3	3.5 - 5	+	+++
B3	33H11	2-10	-	++
B3	UK4	42.5 - 540	-	-
B3	IS4	1120 - 6040	-	-
B3	CL24	2200 - 4920	-	-
IS4	IS4	119.5 - 420	+	-
IS4	B3	4 - 40	+++	-
IS4	CL24	104 - 120	++	-
IS4	UK4	250 - 675	-	-
IS4	33HII	27 - 235	+	-
33H11	33H11	8 - 17.5	-	-
33H11	IS4	24 - 73.5	-	-
33H11	B3	26 - 74	-	-
33H11	UK4	17 - 63.5	-	-
No DNA	No DNA	0	-	-

Table 4.2 Range of IgG concentrations produced in ng/ml and binding characteristics of each heavy/light chain construct during the expression experiments

Abbreviations: - CL – cardiolipin. "+" represents binding of the IgG heavy/light chain combination to the antigen whilst "-" represents no binding.

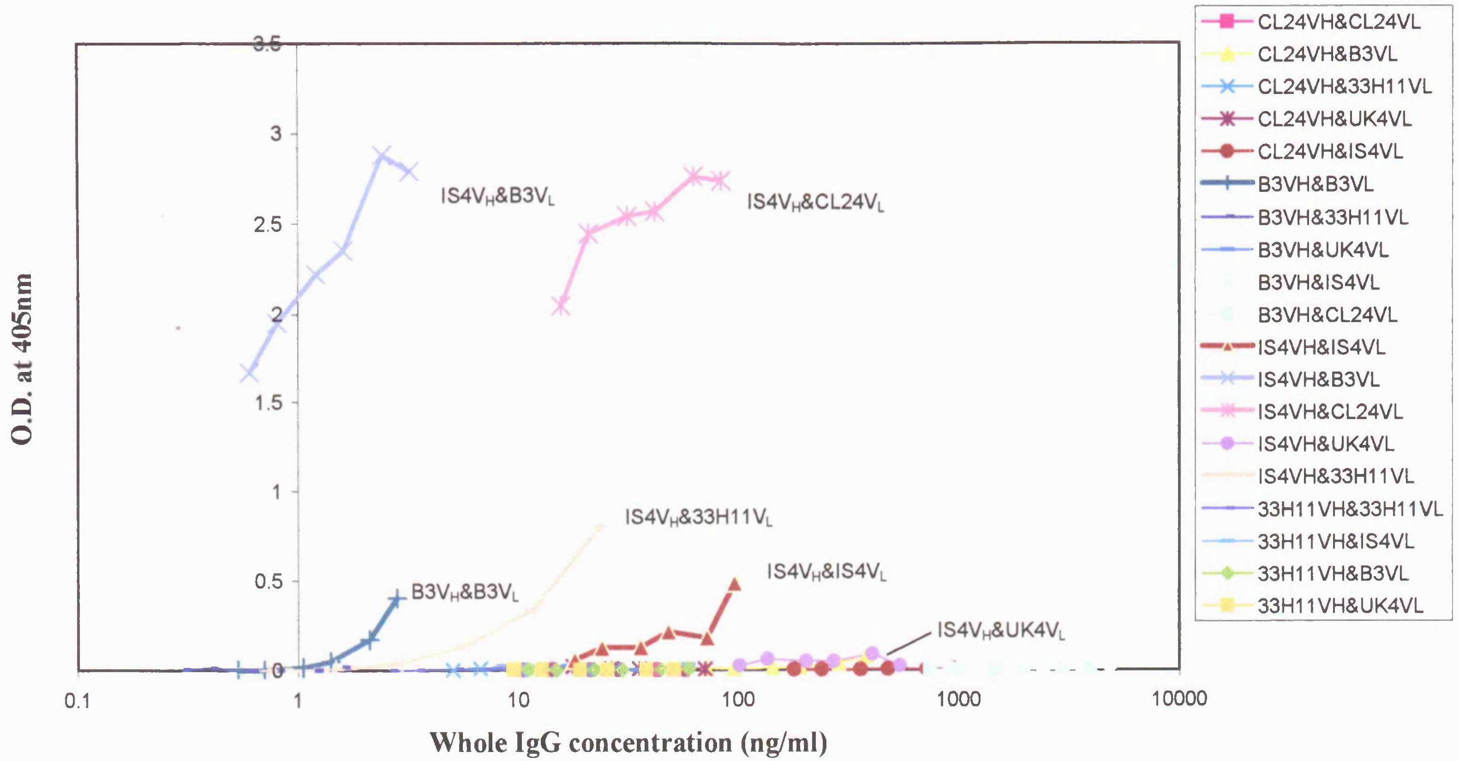


Figure 4.9 A) Results of aCL ELISA

The graphs show binding of IgG in COS-7 cell supernatants containing each heavy light chain combination to cardiolipin.

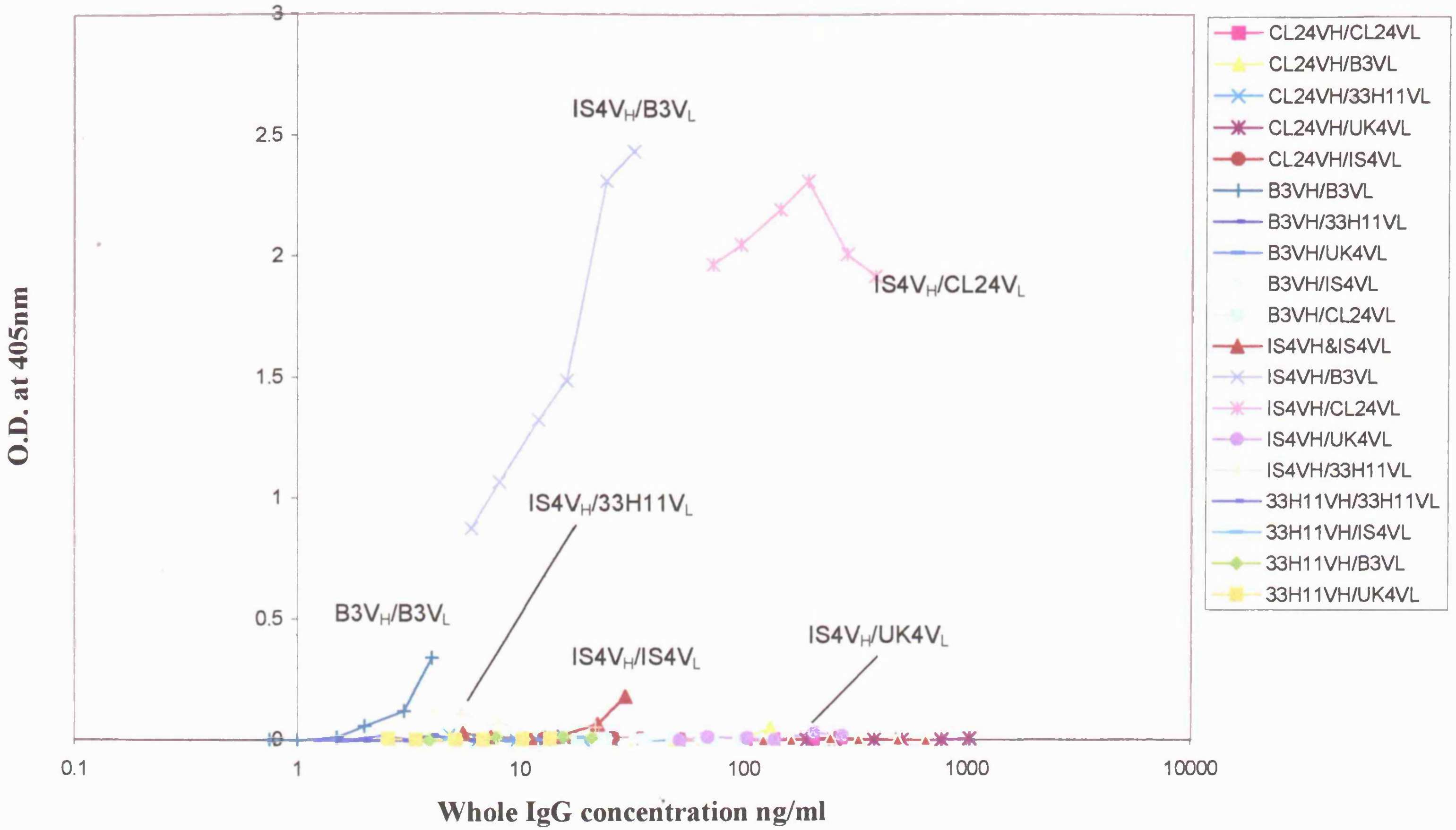


Figure 4.9 B) Results of aCL ELISA

For footnote see Figure 4.9 A).

IS4V_H binds CL in combination with four of the five light chains tested, but these light chains do not bind CL in combination with the other heavy chains tested (with the single exception of B3V_H/B3V_L). This finding suggests that the heavy chain plays a dominant role in binding of IS4 to CL. The differences between the abilities of these four combinations to bind to CL must arise from the differences between their light chains. The wild type combination IS4V_H/IS4V_L did not show the strongest binding to CL. The ability of IS4 to bind this antigen was increased considerably by changing the light chain to B3V_L or CL24V_L, and increased less markedly by changing the light chain to 33H11V_L.

Despite being tested at a range of concentrations between 5.6 and 168 times higher than those which gave maximal CL binding for the other combinations containing IS4V_H, IS4V_H/UK4V_L showed no binding to CL.

The combination B3V_H/B3V_L has previously been shown to bind CL by Kumar et al. (Kumar et al., 2000) after expression of Fab from bacteria. This result was confirmed in the current experiment by expression from COS-7 cells.

4.11 Results of modified anti-cardiolipin ELISA

In order to try and demonstrate that aPL binding to CL was serum dependent and required a cofactor, such as β_2GPI, a modified serum free assay was performed upon supernatant harvested from COS-7 cells grown in the absence of serum. The five V_H/V_L combinations listed above which bound CL in the standard CL ELISA were tested and bound just as strongly in the modified assay as in the original assay, see Figure 4.10.

4.12 Results of anti-β_2GPI ELISA

At the concentrations tested, none of these V_H/V_L combinations could be shown to bind human β_2GPI convincingly. A positive control of human serum, from a patient with PAPS bound well in the anti-β_2GPI ELISA, see Figure 4.11.

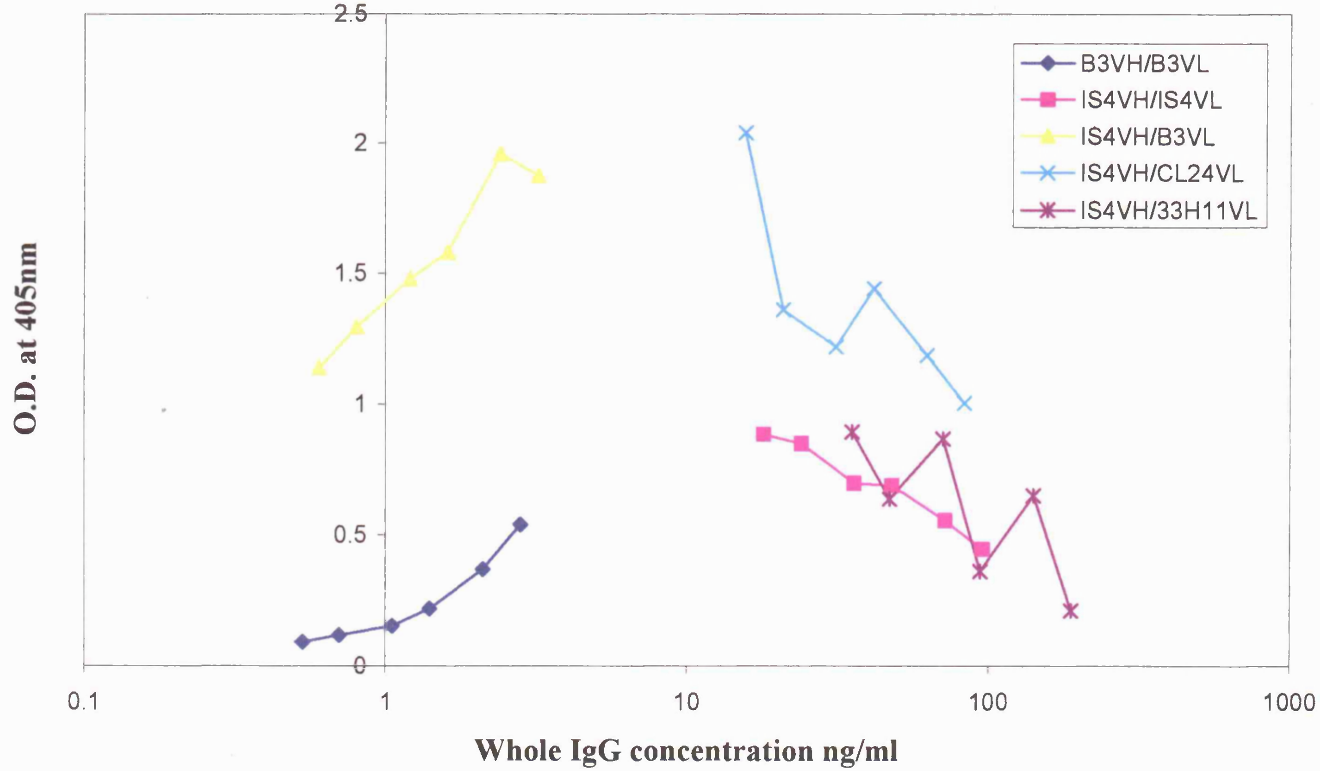


Figure 4.10 Modified aCL ELISA

Cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type heavy chains expressed with wild-type light chain constructs in a modified CL ELISA in which 10% bovine serum albumin was used in place of 10% fetal calf serum.

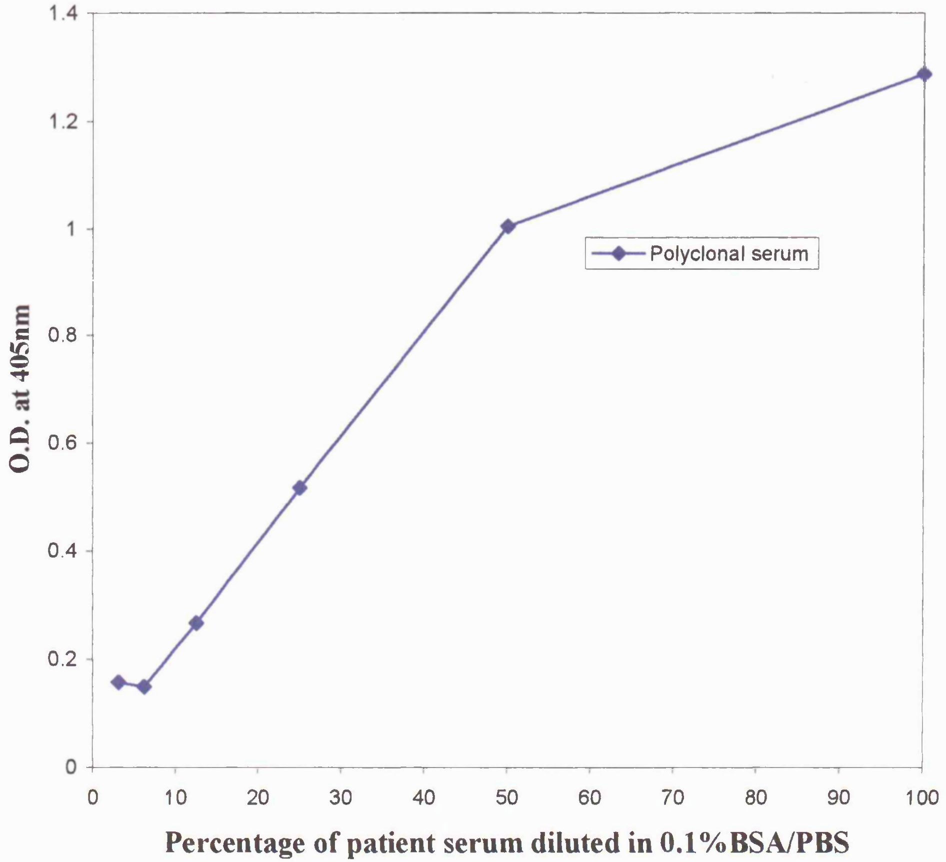


Figure 4.11 Anti-β_2Glycoprotein I ELISA

Patient serum was used as positive control in the anti-β_2Glycoprotein I ELISA, as this gave predictable results in each assay. The values on the X axis display doubling dilutions of neat serum from 100%, in 0.1% bovine serum albumin (BSA)/phosphate buffered saline (PBS).

4.13 Results of anti-DNA ELISA

Each of the V_H/V_L combinations was also examined for binding to dsDNA and consistent patterns of binding were found from successive expression experiments. For each combination, representative results are shown from two separate experiments in Figure 4.12 A) and B).

Only two combinations bound to dsDNA. These were B3V_H/B3V_L and B3V_H/33H11V_L. Both of these heavy/light chain pairs produced very small amounts of whole IgG (combined range of 2-10ng/ml, see Table 4.2) yet clearly bound dsDNA. B3V_H/B3V_L bound dsDNA more strongly than did B3V_H/33H11V_L.

4.14 Modelling of three-dimensional structures

In addition to the modelling studies described in chapter 3.6, B3V_L was also modeled by Dr Sylvia Nagl and is shown in Figure 4.13 alongside IS4V_H, IS4V_L and CL24V_L. The combinations IS4V_H/B3V_L and IS4V_H/CL24V_L displayed the strongest binding to CL (shown in Figure 4.9), whilst the wild-type combination IS4V_H/IS4V_L was much weaker at binding CL.

The models show that multiple surface exposed Arg residues are prominent features of the chains that confer particularly high ability to bind CL. In B3V_L there are four surface exposed Arg residues, three of which have arisen due to somatic mutation, at positions 27 and 27a in CDR1 and position 96 in CDR3, as shown in Figures 4.13 A) and B). The Arg residue at position 54 in CDR2 is germline encoded. CL24V_L has two surface exposed somatically mutated Arg residues in CDR1 and germline encoded Arg residues in CDR2 and FR3, see Figure 4.13 C), whilst IS4V_L has only one surface exposed germline encoded Arg residue in CDR2 (Figure 4.13 D). In the Arg rich CDR3 of IS4V_H, four out of five Arg residues are predicted by the model to be surface exposed and therefore potentially important in binding to cardiolipin, shown in Figures 4.13 E) and 4.13 F).

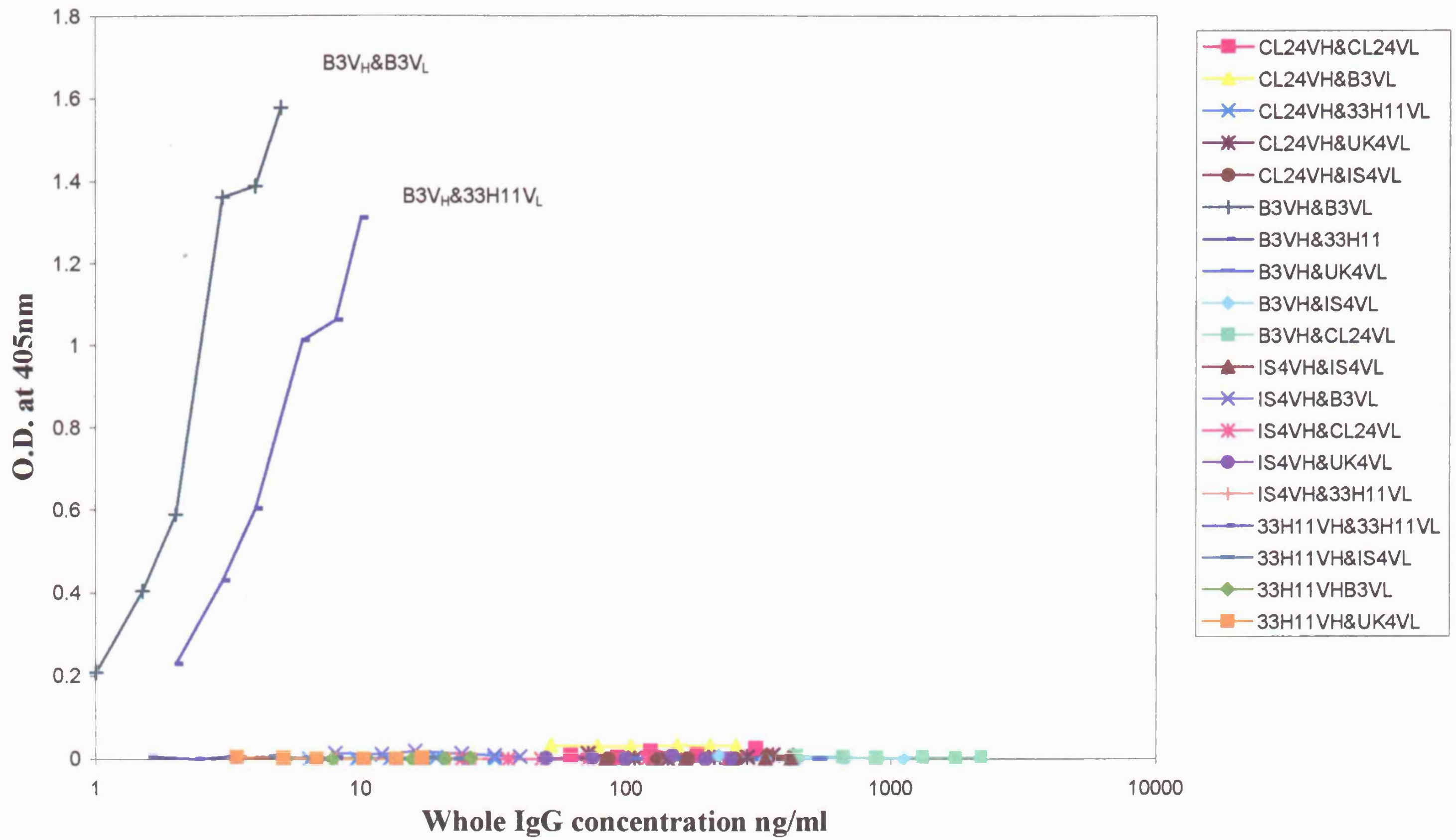


Figure 4.12 A) Results of anti-dsDNA ELISA

Binding of IgG in COS-7 cell supernatants containing each heavy light chain combination to dsDNA.

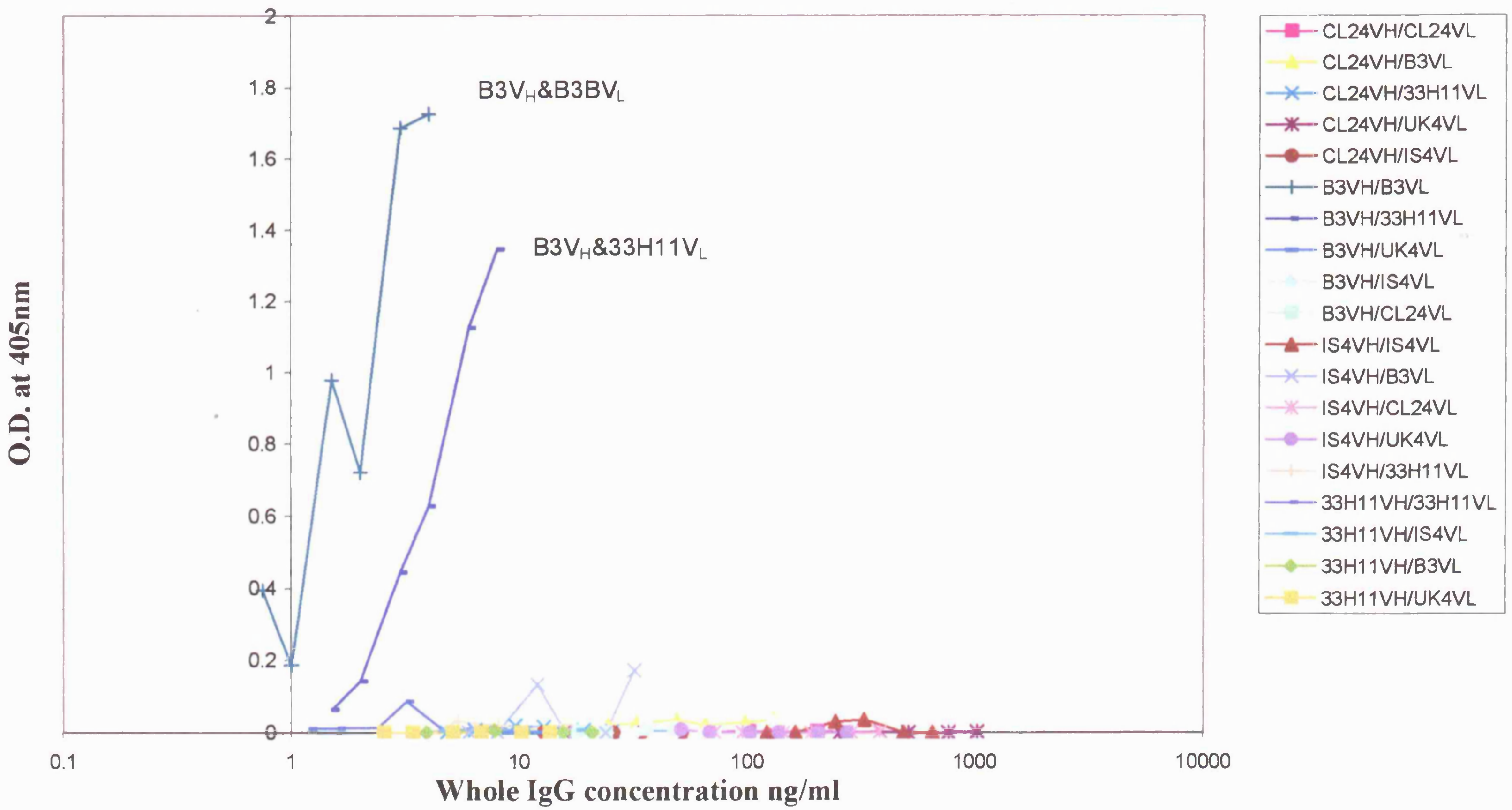


Figure 4.12 B) Results of anti-dsDNA ELISA

Binding of IgG in COS-7 cell supernatants containing each heavy light chain combination to dsDNA.

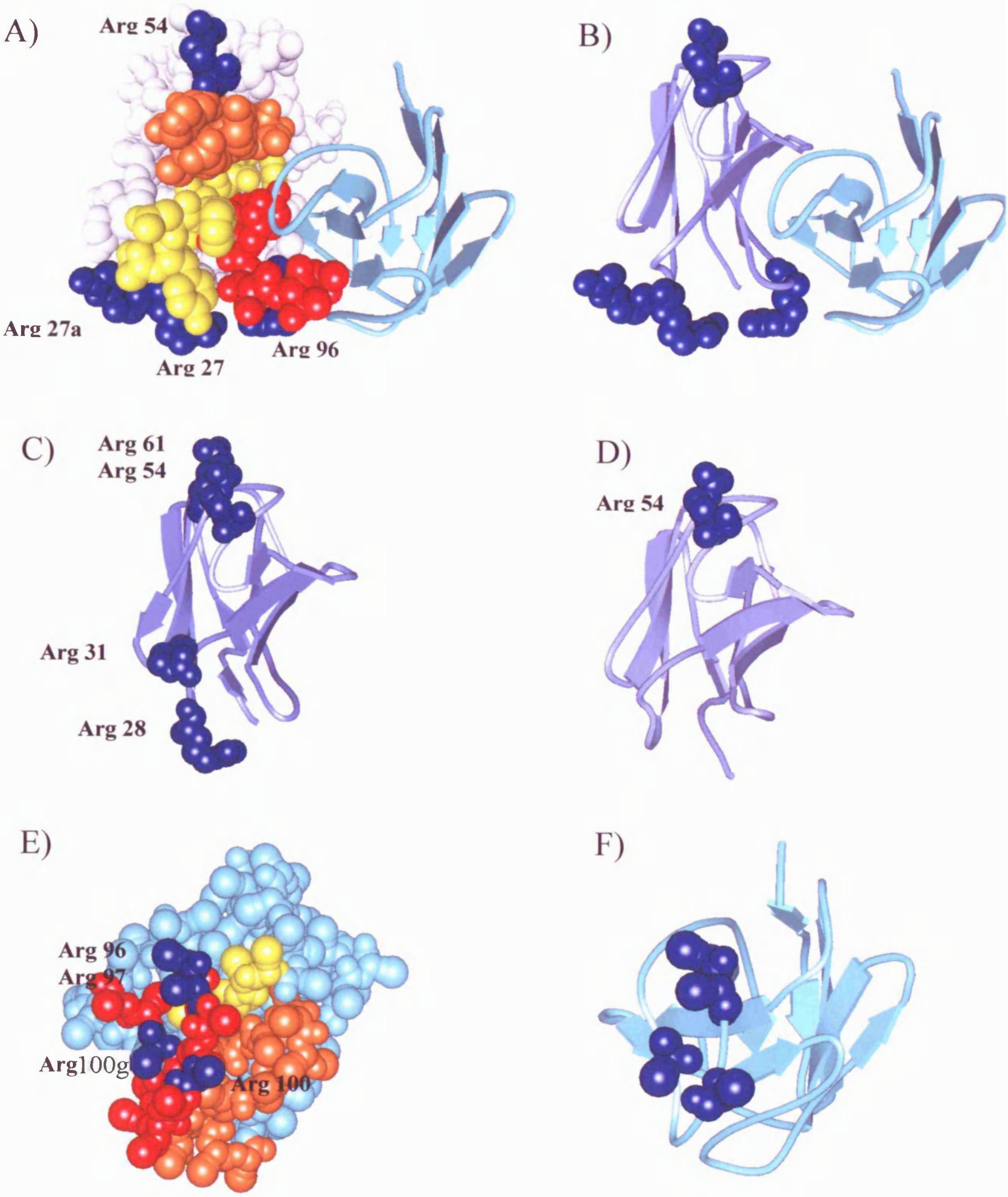


Figure 4.13 Modelling studies of B3V$_L$, CL24V$_L$, IS4VL and IS4V$_H$

B3V$_L$ in A) spacefill mode and B) secondary structure mode. C) CL24V$_L$ in secondary structure mode. D) IS4V$_L$ in secondary structure mode. IS4V$_H$ in E) spacefill mode and F) in secondary structure mode. For abbreviations and colour code see Figure 3.9.

4.15 Discussion of results

The results in this section show that the heavy chain of the human aPL IS4 is dominant in conferring the ability to bind CL and the identity of the light chain is important in fine tuning the strength of that binding. Each of the heavy and light chains that enhanced CL binding contained a number of surface exposed Arg residues most of which are likely to have arisen as a result of antigen drive.

These experiments report the use of an *in-vitro* eukaryotic expression system to produce whole human IgG aPL. Of the two wild-type combinations expressed, IS4V_H/IS4V_L could be shown to bind CL, whereas CL24V_H/CL24V_L could not be shown to bind CL. These results are consistent with the original published data (Zhu et al., 1999). These authors tested affinity purified monoclonal aPL (secreted by hybridoma cells) at much higher concentrations than those I was able to obtain by expression in COS-7 cells. They found that IS4 bound more strongly to CL than CL24. Twice as much CL24 (2μg/ml) as IS4 (1μg/ml) was required to obtain similar binding to CL in ELISA. The amounts of CL24V_H/CL24V_L produced by the COS-7 cells (69.5-310ng/ml) in these experiments were six times lower than the levels of CL24 originally tested (Zhu et al., 1999). The lack of binding of the expressed CL24V_H/CL24V_L in the aCL ELISA is therefore likely to be due to the low concentration of antibody tested.

The great advantage of this transient expression system is that it allows rapid testing of large numbers of heavy/light chain combinations. The results of these experiments allow inferences to be made about the importance of particular sequence motifs in binding to DNA and CL. Because IS4V_H utilises an unusual germline gene (V1-03) in which the pattern of somatic mutations are not suggestive of antigen drive, these properties are unlikely to account for its dominant role in binding to CL. The most likely contributor to the CL-binding specificity of IS4V_H is its highly mutated CDR3 region which contains five Arg residues, four of which are surface exposed, see Figure 4.13 E) and F) and could therefore be important in contributing to CL-binding specificity. It seems likely that the selecting antigen was CL or a complex containing CL, since the presence of IS4V_H tends to confer the ability to bind this antigen.

Although the IS4V_H sequence plays a dominant role in conferring the ability to bind CL, the nature of the light chain paired with it affects the strength of binding. The B3V_L and CL24V_L sequences are much more favorable for binding of CL than IS4V_L. A striking similarity between the sequences of B3V_L and CL24V_L is that both have two Arg residues created by somatic mutation in CDR1. In contrast, IS4V_L has no somatic mutations to Arg. The modelling studies in Figures 4.13 (A-C) clearly show that the somatically mutated Arg residues in CDR1 of B3V_L and CL24V_L are surface exposed. These somatic mutations may confer an additional ability to bind to CL above that conferred by the germline encoded Arg at position 54 which is present in all three of those light chains.

I was unable to demonstrate binding of either IS4V_H/IS4V_L or CL24V_H/CL24V_L produced from COS-7 cells to ß$_2$GPI. Both these antibodies were originally described as weak binders to human ß$_2$GPI, requiring testing at concentrations of 10 to 20µg/ml of purified antibody, in order to demonstrate any binding on ELISA (Zhu et al., 1999). It is therefore unsurprising that it was not possible to detect binding to ß$_2$GPI using the much smaller amounts of antibody (119.5-420ng/ml of IS4V_H/IS4V_L and 69.5-310ng/ml of CL24V_H/CL24V_L) obtained using this expression system.

An indirect method of demonstrating that these antibodies bound ß$_2$GPI would have been to show that their binding to CL could be abolished in the absence of serum. I was unable to demonstrate this by growing the COS-7 cells in serum free media post electroporation and subsequent testing in a modified and serum, hence ß$_2$GPI free CL ELISA. The most likely reason for this is that ß$_2$GPI was adsorbed onto the surface of the COS-7 cells during their growth in medium containing serum, prior to transfection, and that this ß$_2$GPI was released into the supernatant after some of these cells were killed during electroporation. Possible measures to resolve this problem would be to grow the cells in serum free medium throughout, or to affinity purify the antibodies from the supernatant before testing. Affinity purification is not feasible with the small amounts of antibody produced from COS-7 cells. However, these measures became unnecessary following successful demonstration of direct binding of the antibodies to ß$_2$GPI at the higher concentrations expressed in chapter five.

Low levels of expression would explain the lack of binding seen with 33H11V_H/33H11V_L in the anti-dsDNA ELISA. Much smaller amounts of antibody were produced by this expression system (8-17.5ng/ml) compared to that produced by hybridoma cells (3-35μg/ml) in the original experiments which characterized binding to dsDNA (Winkler et al., 1991). These results were consistent with those obtained in previous studies of expression of this combination in COS-7 cells (Rahman *et al.*, 1998; Rahman *et al.*, 2001) and as Fab from bacteria (Kumar et al., 2000).

I studied binding of the expressed antibodies both to CL and to dsDNA. The links between antibodies binding to these two antigens are a source of some interest. Anti-dsDNA antibodies and aPL share a number of sequence characteristics and some show dual specificity for both antigens (Kumar *et al.*, 2000; Cocca *et al.*, 2001). It has been shown that a murine anti-PC antibody can lose specificity for phospholipid and become an anti-DNA antibody as the result of a single point mutation in V_HCDR3 (Diamond and Scharff, 1984).

Furthermore, it seems increasingly likely that both aPL and anti-dsDNA antibodies may arise due to an immune response directed against debris from apoptotic cells. Cocca *et al.* (2001) showed that a particular pattern of mutations to arginine in V_H of the murine antibody 3H9 could enhance binding to PL, dsDNA and to blebs derived from apoptotic cells. Conversely, Pewzner-Yung *et al.* (1996) showed that some sequence features in the V_H sequences of murine antibodies derived from the same germline gene, V_H 11, enhanced affinity for DNA whereas different features favoured binding to PC.

Some of the human heavy and light chains that I expressed were derived from aPL whereas others were derived from anti-DNA. By studying the properties of various combinations of these chains, I aimed to distinguish sequence features important in binding to DNA from those important in binding to PL.

Previous experiments have shown that Arg at position 27a in B3 V_L CDR1 plays an important role in binding to dsDNA (Rahman et al., 2001). This may be an example of an Arg residue which, like those described by Cocca *et al.* (2001), can enhance binding to both DNA and PL. In contrast, the Arg residue in CDR3 (Arg92) of 33H11V_L, which enhances binding to DNA (Rahman et al., 2001), does not seem to enhance binding to CL as much as the CDR1 Arg residues in B3V_L and CL24V_L. The combination IS4V_H/33H11V_L did not bind CL as well as either IS4V_H/B3V_L or IS4V_H/CL24V_L.

The presence of UK4V_L appears to be inhibitory because it was the only light chain which did not bind CL in combination with IS4V_H. Previously a computer model has shown that the inhibitory effect of UK4V_L upon dsDNA binding is likely to be due to a large blocking Arg group at position 94 (Arg94) of UK4V_L CDR3 (Rahman et al., 2001). It is possible that the inhibitory effect of UK4V_L upon binding to CL observed in this study is due to the same Arg94 residue.

The Arg-rich CDR3 sequence of IS4V_H does not confer the ability to bind dsDNA. This heavy chain did not bind DNA in combination with any of the five light chains tested, despite the fact that two of them (B3V_L and 33H11V_L) are derived from anti-DNA antibodies and can bind DNA when expressed with B3V_H in this system.

The COS-7 cell expression system has previously been used to express B3V_H in combination with light chains derived from four different monoclonal antibodies, including B3 itself (Rahman *et al.*, 1998; Rahman *et al.*, 2001). Although whole IgG molecules were obtained from each combination, only two combinations bound dsDNA in ELISA. The new combinations B3V_H /IS4V_L and B3V_H /CL4V_L do not bind dsDNA either. These results support the contention that the presence of an appropriate light chain is crucial in enabling antibodies containing B3V_H to bind DNA (Rahman et al., 2001) and (Kumar et al., 2000). Computer modelling studies suggested that Arg27a in B3V_L CDR1 and Arg 92 in 33H11V_L CDR3 form important interactions with the dsDNA molecule (Rahman et al., 2001). It is clear that the exact positions occupied by these Arg residues are important since UKV_L and CL24V_L will not bind dsDNA in combination with any heavy chain tested, despite containing Arg in CDR3 and CDR1 respectively.

In summary, I have described the expression of whole human aPL molecules from cloned cDNA *in-vitro*. I have used this system to demonstrate the importance of Arg residues in both heavy and light chains in binding to CL or dsDNA. Whereas Arg residues at some sites (e.g. position 27a of B3 V_L CDR1) may play a role in binding to both these antigens, there are other positions at which Arg enhances binding to one antigen or the other, but not both. For example, the multiple Arg residues in IS4V_HCDR3 enhance binding to CL but not dsDNA. Finally, some Arg residues, such as Arg 94 in UK4V_L, seem to inhibit binding to both antigens. The presence and exact location of Arg residues in the CDRs play a major role in binding to CL, which is different to the role played by these residues in creating the DNA binding site.

Therefore the next step was to use the transient expression system to further characterise the role of specific Arg residues in both heavy and light chains of these whole human aPL. This work was performed by swapping CDRs between 2a2 gene encoded light chains (IS4, UK4 and B3) and pairing the variant chains with IS4V_H and B3V_H. Thus the overall structure of each chain is retained but the pattern of mutations is altered. As a result of such CDR exchange one may investigate whether the Arg residues in B3V_L CDR1 lead to an enhanced binding to CL when combined with IS4V_L CDR2 and 3 and whether the suggested blocking effect of the Arg in UK4 CDR3 is dominant. In addition site directed mutagenesis of specific Arg codons at sites of antigen contact, e.g. in IS4V_H CDR3, to germline residues were performed to assess the contribution of individual Arg residues to CL binding.

CHAPTER 5

SOMATIC MUTATIONS TO ARGININE RESIDUES AFFECT THE BINDING OF HUMAN MONOCLONAL ANTIBODIES TO CARDIOLIPIN

5.1 Generation of hybrid light chains

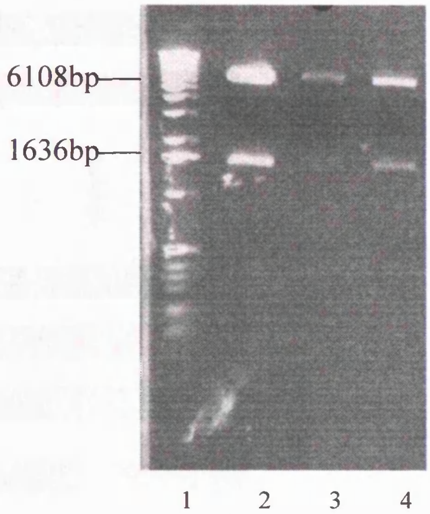
Hybrids were made from three parental light chains: - B3V_L; UK4V_L; and IS4V_L. The hybrids were named by combining the names of the two parent antibodies such that the first letter represented the antibody from which the CDR1 was derived and the last letter represented the antibody from which both the CDR2 and 3 were derived. Thus, hybrid IB contains CDR1 from IS4 and CDR2 and 3 from B3 whereas hybrid BI contains the reverse combination (CDR1 from B3 and CDR2 and 3 from IS4). All three light chains are encoded by the 2a2 gene and differ only in their pattern of somatic mutation. The restriction fragments produced by *Acc*65 I and *Pvu* I digest of expression plasmids containing these V_L sequences are shown in Table 5.1 and Figure 5.1.

Light chain donating CDR1 (parent plasmid & fragment size after *Acc*65 I/*Pvu* I digest)	Light chain donating CDR2 & CDR3 (parent plasmid & fragment size after *Acc*65 I/*Pvu* I digest)	Hybrid (overall size)
IS4 (pLN100, 6323bp)	B3 (pLN10, 1534bp)	IB (7857bp)
IS4 (pLN100, 6323bp)	UK4 (pLN10, 1528bp)	IU (7851bp)
B3 (pLN10, 5963bp)	IS4 (pLN100, 1534bp)	BI (7497bp)
UK4 (pLN10, 5963bp)	IS4 (pLN100, 1534bp)	UI (7497bp)

Table 5.1 Components of each hybrid

5.2 Confirmation digest of 2a2 V_L hybrids

To confirm the production of the required hybrid, I took advantage of the fact that the patterns of internal *Ava* I and *Aat* II sites difer between the three parental light chains as shown in Figure 5.2. Thus, for example, the hybrids IB and BI can be distinguished from both parental sequences because IB contains no *Aat* II sites whereas BI contains two *Aat* II sites.



Lanes
1 = 1Kb marker
2 = *Acc*65 I /*Pvu* I digested IS4V_L/pLN100
3 = *Acc*65 I /*Pvu* I digested UK4V_L/pLN10
4 = *Acc*65 I /*Pvu* I digested B3V_L/pLN10

Figure 5.1 *Acc*65 I/*Pvu* I digest for creation of 2a2 hybrid light chains

Acc 65 I/*Pvu* I digest of IS4V_L/pLN100 (1534bp and 6323bp) in lane 2; UK4V_L/pLN10 (1528bp and 5963bp) in lane 3; and B3V_L/pLN10 (1534bp and 5963bp) in lane 4. The smaller fragment in lanes 2 – 4 contains CDR2 and CDR3 of each light chain variable region whilst CDR1 remains in the larger fragment.

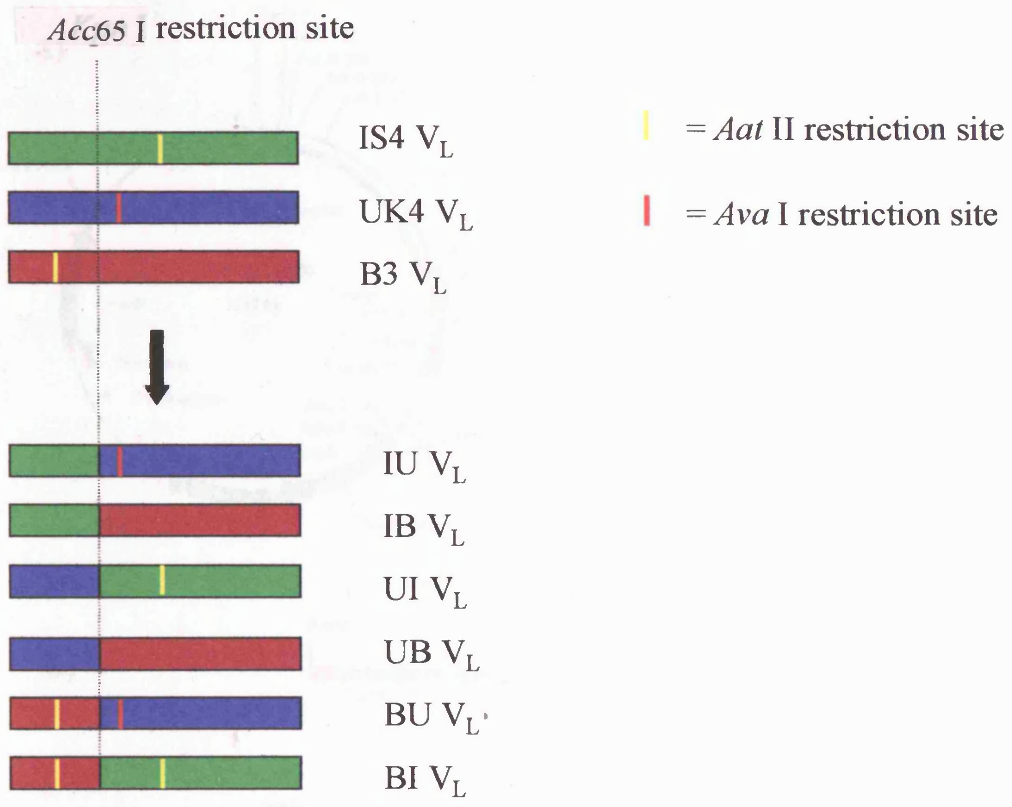


Figure 5.2 Altered patterns of *Aat* II and *Ava* I restriction sites in 2a2 V_L hybrids

Schematic representation of *Aat* II and *Ava* I restriction sites in hybrid (CDR exchanged) light chains compared to the native light chains from which they were derived, B3V_L; UK4V_L; and IS4V_L. An *Aat* II site occurs at position 158 in IS4V_L CDR2 and position 78 in B3V_L CDR1. UK4V_L contains an *Ava* I site at position 115 in FR2. All native and hybrid light chains contain *Acc*65 Isite at position 106.

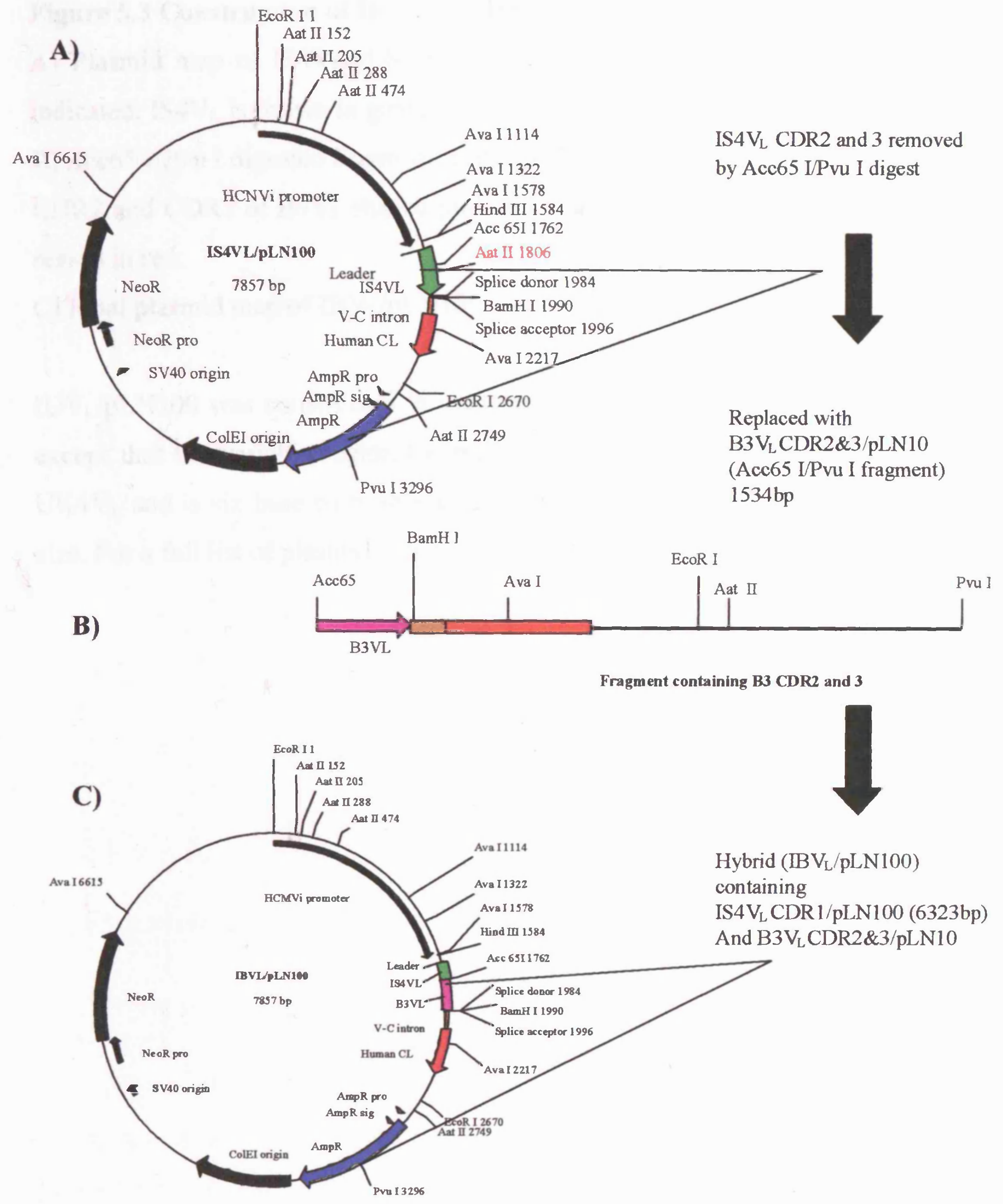


Figure 5.3 Construction of IBV$_L$/pLN100 hybrid

For legend see following page.

Figure 5.3 Construction of IBV_L/pLN100 hybrid

A) Plasmid map of IS4V_L/pLN100 with the region removed by a *Acc*65 I/*Pvu* I digest indicated; IS4V_L is shown in green and the human C_L region in red.

B) *Acc*65 I/*Pvu* I digested fragment from B3VL/pLN10 is shown with the region containing CDR2 and CDR3 of B3V_L shaded pink, the introns shaded brown and the human constant region in red.

C) Final plasmid map of IBV_L/pLN100.

IUV_L/pLN100 was constructed in the same way and is almost identical to IBV_L/pLN100 except that the variable region fragment containing CDR2 and CDR3 was donated from UK4V_L and is six base pairs shorter than that of B3V_L, hence altering the overall plasmid size. For a full list of plasmid abbreviations see Figure 2.5.

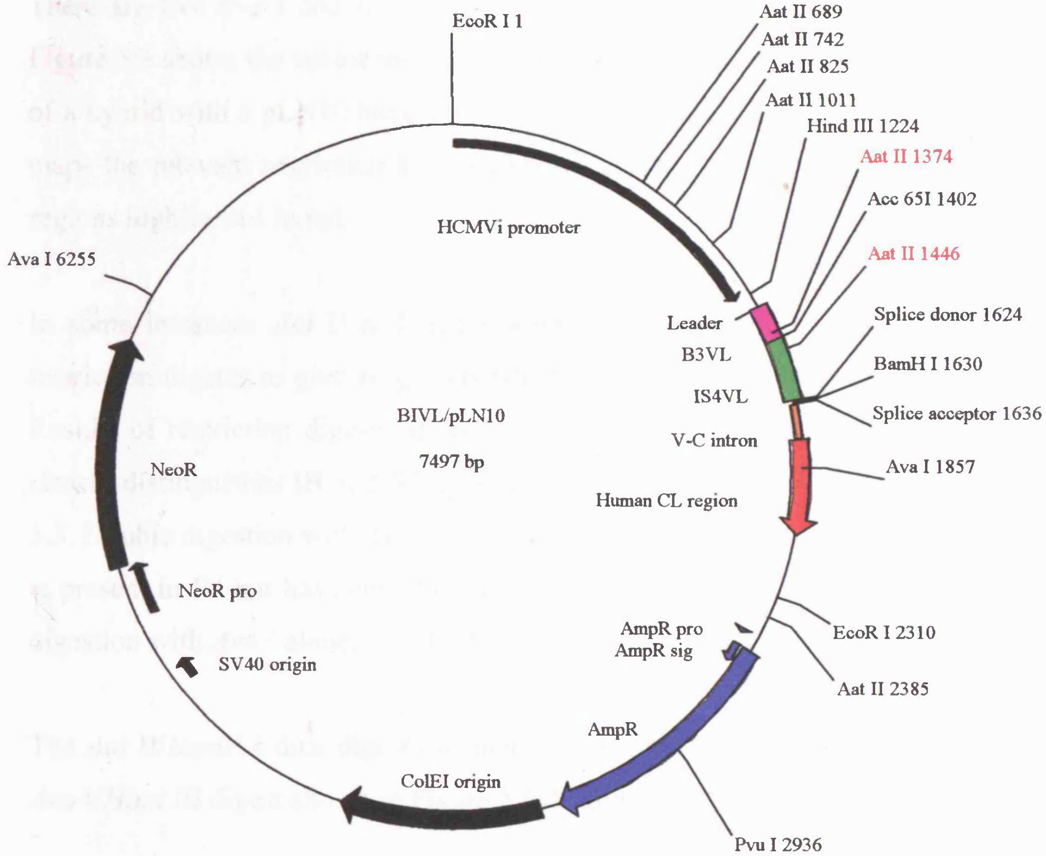


Figure 5.4 Plasmid map of BIVL/pLN10 hybrid

Plasmid map of BIV$_L$/pLN10 with the region containing B3V$_L$CDR1 shown in pink and IS4V$_L$ CDR 2 and CDR3 shown in green, the intron is shaded brown and the human constant region red. The construction of UIV$_L$/pLN100 is similar to BIV$_L$/pLN100 except that the fragment containing B3V$_L$CDR1 is replaced by UK4V$_L$ and the overall size of the plasmid is the same. For a full list of plasmid abbreviations see Figure 2.7.

There are five *Ava* I and five *Aat* II sites in the expression vectors pLN100 and pLN10. Figure 5.3 shows the vector map of hybrid IB and its construction whilst a final vector map of a hybrid with a pLN10 backbone is shown in Figure 5.4. In each of these hybrid vector maps the relevant restriction sites are shown and the distinguishing *Aat* II sites in the V_L regions highlighted in red.

In some instances *Aat* II and *Ava* I were combined with either *Hind* III or *Bam* HI in restriction digests to give fragments which were easily distinguishable on an agarose gel. Results of restriction digests of are shown in Figures 5.5 to 5.8. The *Aat* II digest alone clearly distinguishes IB and IU hybrids from the parent plasmid IS4V_L/pLN100 in Figure 5.5. Double digestion with *Ava* I/*Hind* III identifies IB from IU, in Figure 5.6 because *Ava* I is present in IU but has been removed in IB. Double digestion was carried out rather than digestion with *Ava* I alone, in order to create fragments of easily distinguishable lengths.

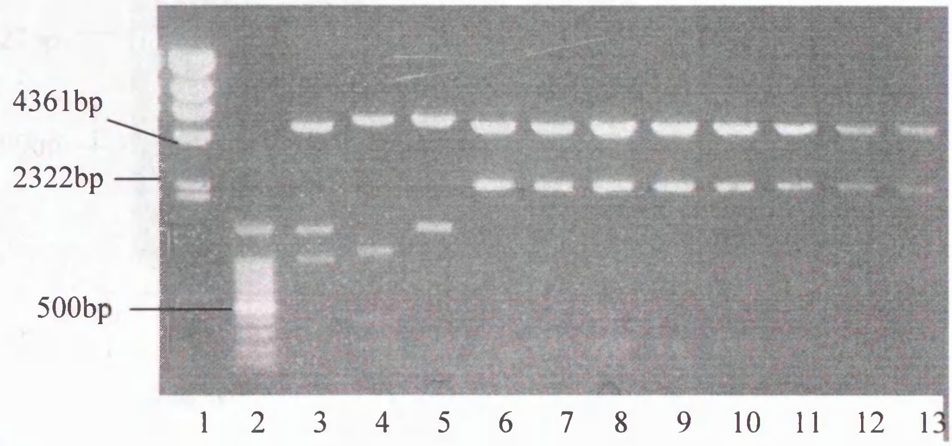
The *Aat* II/*Bam*H I dual digest distinguishes BI from B3V_L/pLN10, see Figure 5.7 and the *Ava* I/*Hind* III digest shown in Figure 5.8 distinguishes UI from UK4V_L/pLN10.

5.3 Site directed mutagenesis of IS4V_H

Six mutant forms of IS4V_H were created by site directed mutagenesis and plasmid DNA of the appropriate size was confirmed, post transformation, by a Hind III/Xho I digest. This plasmid DNA was then sent for sequencing (MWG-Biotech) which confirmed the presence of the desired point mutations in each heavy chain.

5.4 Heat shock and Phospholipase C treatment of COS-7 cell supernatants

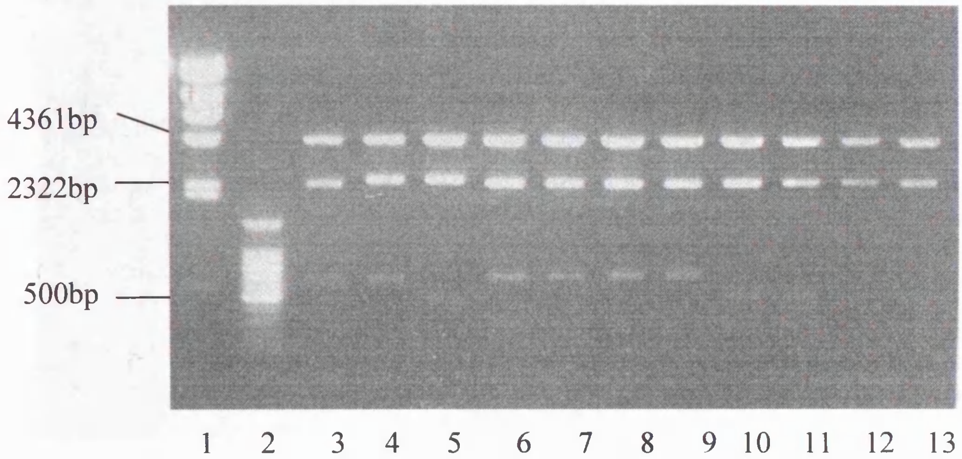
During electroporation approximately 80% of the cells die, releasing their contents (including DNA and PL) into the supernatant. Anti-dsDNA antibodies produced by this expression system have been shown to bind to DNA in the supernatant. Treatment of these COS-7 cell supernatants containing DNA binding antibodies with DNase I prior to their testing in a whole IgG ELISA led to a relative increase in the amount of antibody detected in the whole IgG assay and was essential in order to detect these antibodies in an anti-DNA ELISA (Rahman et al., 1998). The DNase I was considered to have broken down mAb-DNA immune complexes in the supernatant leaving the IgG molecules free to be detected in the supernatants.



Lanes
1 = Lambda DNA/*Hind* III marker
2 = 100bp marker
3 = *Aat* II digested IS4V_L/pLN100
4 = *Aat* II digested B3V_L/pLN10
5 = *Aat* II digested UK4V_L/pLN10
6 - 9 = *Aat* II digested IBV_L/pLN100
10 - 13 = *Aat* II digested IUV_L/pLN100

Figure 5.5 Confirmation *Aat* II digest of IB and IU hybrid light chains

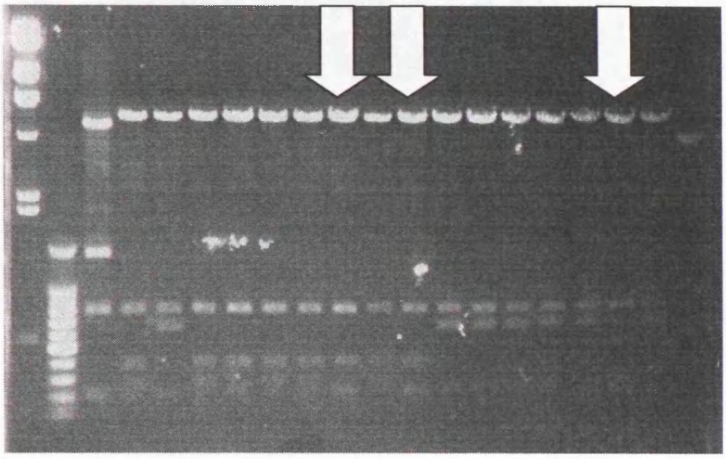
The hybrids IBV_L/pLN100 (lanes 6 – 9) and IUV_L/pLN100 (lanes 10 – 13) are clearly different from the parental vectors. They differ from IS4V_L/pLN100 (lane 3) and B3V_L/pLN10 (lane 4) due to loss of the internal *Aat* II site. They differ from UK4V_L/pLN10 (lane 5) due to a different pattern of *Aat* II sites in the expression vectors pLN10 and pLN100.



Lanes
1 = Lambda DNA/*Hind* III marker
2 = 100bp marker
3 = *Hind* III/*Ava* I digested IS4V_L/pLN100
4 = *Hind* III/*Ava* I digested B3V_L/pLN10
5 = *Hind* III/*Ava* I digested UK4V_L/pLN10
6 - 9 = *Hind* III/*Ava* I digested IBV_L/pLN100
10–13 = *Hind* III/*Ava* I digested IUV_L/pLN100

Figure 5.6 Confirmation *Hind* III/*Ava* I digest of IB and IU hybrid light chains

The hybrids IBV_L/pLN100 (in lanes 6 – 9) and IUV_L/pLN100 (lanes 10 – 13) are easily distinguished from each other due to the presence of an internal *Ava* I site in IUV_L but not IBV_L.



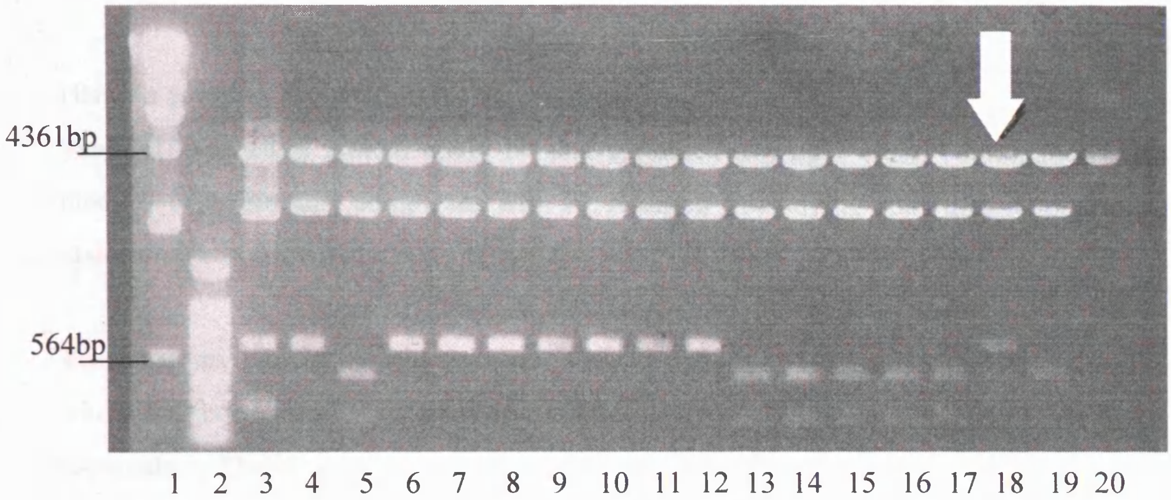
1 - - - 5 - - - - 10 - - - - 15 - - - - 20

Lanes:

1	= Lambda DNA/*Hind* III marker
2	= 100bp marker
3	= *Aat* II/*Bam*H I digested IS4VL/pLN100
4	= *Aat* II/*Bam*H I digested B3VL/pLN10
5	= *Aat* II/*Bam*H I digested UK4VL/pLN10
6 – 12	= *Aat* II/*Bam*H I digested BIVL/pLN10
13 – 19	= *Aat* II/*Bam*H I digested UIVL/pLN10
20	= uncut B3VL/pLN10

Figure 5.7 Screening *Aat* II/*Bam*H I digest of BI and UI hybrid light chain ligations

In the above figure each lane represents plasmid derived from a different colony on LBAgar plates. The expected pattern on restriction digest with *Aat* II/*Bam*H I of the hybrids is seen only in lanes indicated by an arrow; 10 and 12 for BIV_L/pLN100; and lane 18 for UIV_L/pLN100. These differences are seen because the hybrid BIV_L/pLN100 has an additional internal *Aat* II site compared to the parent plasmids B3V_L/pLN10 (lane 4) and IS4V_L/pLN100 (lane 3) and UIV_L/pLN100 has gained an internal *Aat* II site compared to UK4V_L/pLN10.



Lanes:
1 = Lambda DNA/*Hind* III marker
2 = 100bp marker
3 = *Hind* III/*Ava* I digested IS4V_L/pLN100
4 = *Hind* III/*Ava* I digested B3V_L/pLN10
5 = *Hind* III/*Ava* I digested UK4V_L/pLN10
6 - 12 = *Hind* III/*Ava* I digested colonies from BIV_L/pLN100 ligations
12 - 19 = *Hind* III/*Ava* I digested colonies from UIV_L/pLN10 ligations
20 = Uncut B3V_L/pLN10

Figure 5.8 Screening *Hind* III/*Ava* I digest of BI and UI hybrid light chain ligations

In the above figure each lane represents plasmid derived from a different colony on LBAgar plates. The pattern on restriction digest with *Hind* III/*Ava* I with the hybrids BIV_L/pLN100 (successful ligation in lanes 6 – 12) and UIV_L/pLN100 (successful ligation in lane 18 only) will be indistinguishable from that of B3V_L/pLN10 (lane 4) because none of these plasmids contain an internal *Ava* I site, but will clearly differ from UK4V_L/pLN10 (lane 5) because this plasmid does contain an internal *Ava* I site.

Hence a possible explanation for the observed variation in the yield of whole IgG from the transient expression system could be that certain heavy/light chain combinations which bind PL form immune complexes with PL released from dying cells thus rendering them undetectable in ELISA.

Two methods, heat shock and treatment with PLC were tried to see if a relative increase in whole IgG concentration or enhanced binding to CL could be revealed in the COS-7 cell supernatant. Three heavy/light chain combinations were examined; IS4V_H/IS4V_L because of its weak CL binding; IS4V_H/B3V_L because this gave the strongest pattern of CL binding; and B3V_H/IS4V_L to see if any CL binding could be revealed from this non-binder. Samples of each of these three COS-7 cell supernatants were divided into two identical aliquots which were treated with heat shock or PLC. Figure 5.9 displays the graphs of whole IgG concentration of these heavy/light chain combinations remaining identical both pre and post heat shock and treatment with PLC. The effects of these treatments upon CL binding are shown in Figure 5.10 and there is clearly no difference with the IS4V_H/IS4V_L and B3V_H/IS4V_L combinations. The CL binding of IS4V_H/B3V_L however was reduced by approximately a third when treated with heat shock or PLC (both the active and inactive aliquots). Therefore there was no evidence that immune complex formation is masking detection of aPL from any of these combinations.

5.5 Comparison of three and six day harvest from COS-7 cell supernatant

In the transient expression experiments described in chapter 3, the supernatants of the transfected cells were collected after 72 hours (when fresh medium was applied) and 144 hours of incubation in 5% CO_2 at 37°C. The reason for this dual harvest was that previously this same expression system has been shown to continue to express whole IgG for at least six days post electroporation (Haley, 2003).

To investigate whether COS-7 cells transfected with the heavy/light chain combinations described in this thesis continue to express IgG for longer than three days the concentration of expressed IgG was measured from the three and six day harvest separately for comparison. Each harvest was treated identically by treatment with DNase I and sodium azide before the concentration of IgG was determined by ELISA (as in section 2.18.1).

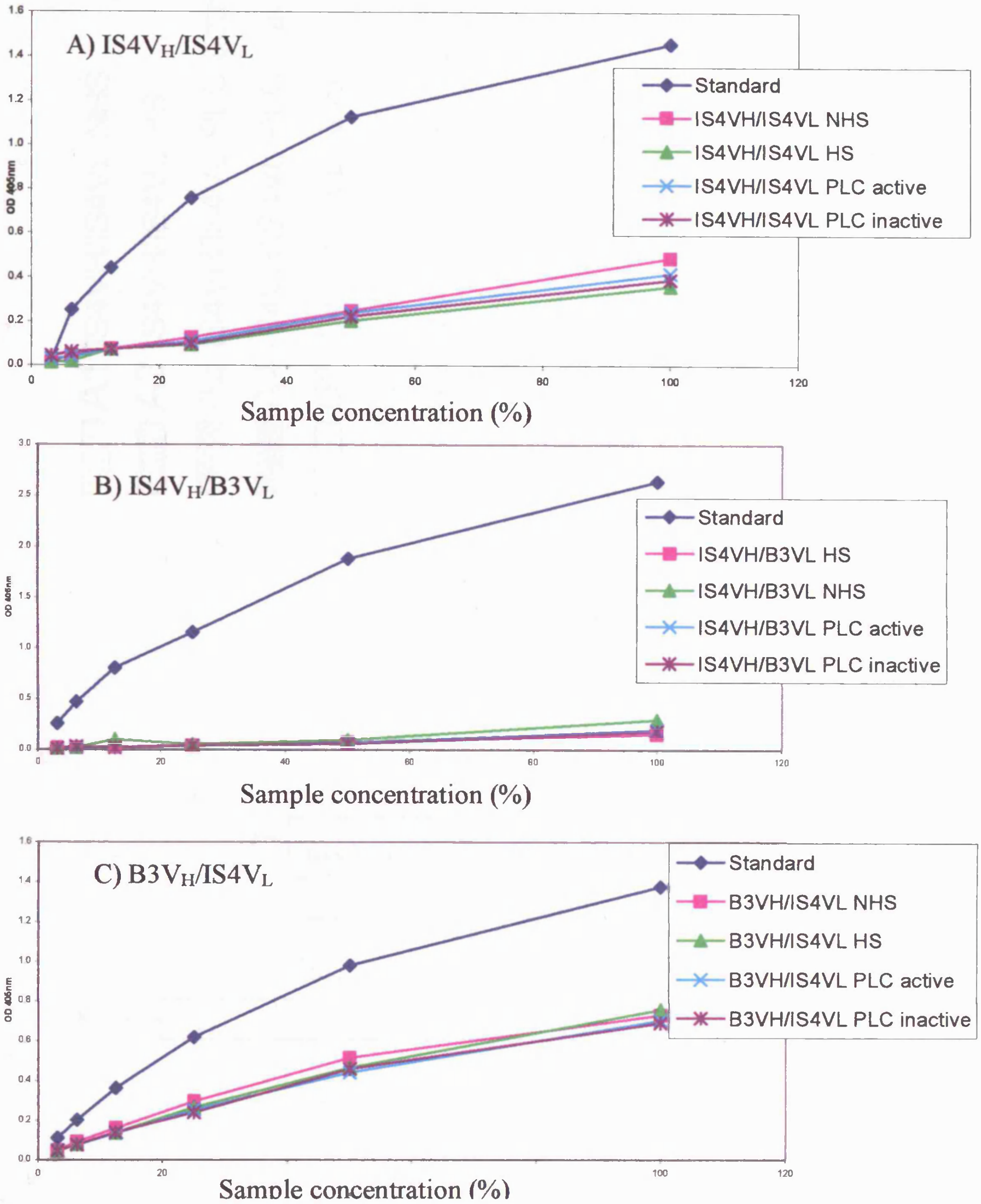


Figure 5.9 Effects of heat shock and Phospholipase C upon IgG concentration of three heavy/light chain combinations

Each graph shows OD (405nm) on the y axis against serial dilutions of a standard (human IgG_{λ}, 100% concentration = 100ng/ml) and four heavy/light chain combinations upon an ELISA plate. Concentration of IgG in the latter is estimated by comparison with the standard. Abbreviations: - NHS – no heat shock; HS – heat shock; PLC – Phospholipase C.

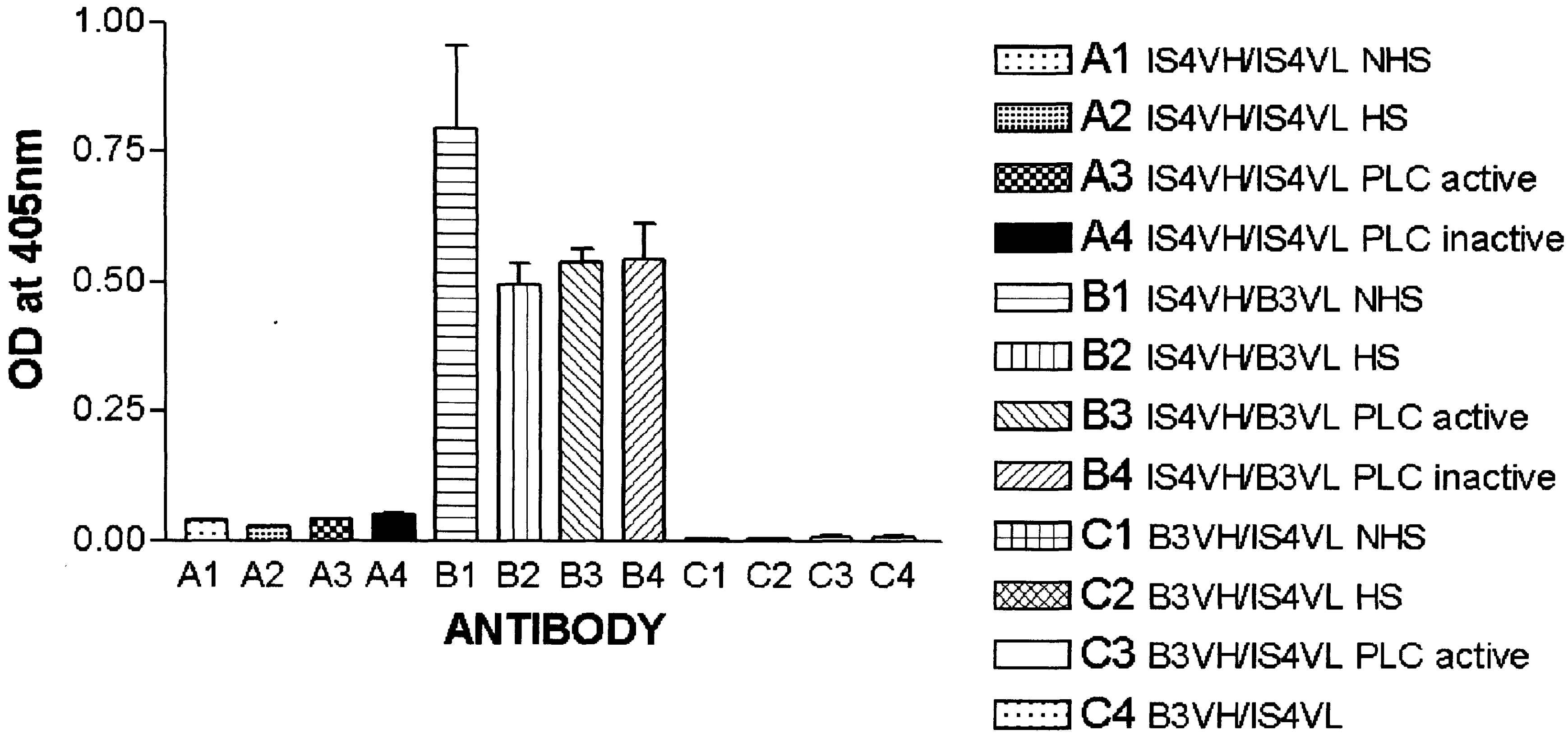


Figure 5.10 Effects of heat shock and phospholipase C upon cardiolipin binding of three heavy/light chain combinations

Each column represents an average OD (+/- Standard Deviation) taken from the six different aliquots of each antibody.

Abbreviations: - NHS – no heat shock; HS – heat shock; PLC – phospholipase C.

Table 5.2 shows that of the 12 heavy/light chain combinations tested five did not produce any further IgG at all after three days, and four combinations produced less than 10ng/ml in the same period. Only three combinations continued to produce an appreciable amount of IgG at the six day harvest produce. Thus when harvesting expressed IgG from heavy/light chain combinations containing point mutations in either chain, supernatants were collected after three days and the cells were then discarded.

ANTIBODY		CONCENTRATION ng/ml	
Heavy chain	Light chain	3/7 harvest	6/7 harvest
IS4	IS4	22	6
IS4	B3	10	0
IS4	UK4	10	0
IS4	IB	16	4
IS4	IU	30	6
IS4	BU	20	0
IS4	UB	20	0
B3	IS4	45	36
B3	UK4	10	0
B3	IB	75	32
B3	IU	110	40
B3	BU	39	<3.125
No DNA	No DNA	0	0

Table 5.2 IgG concentrations in COS-7 cell supernatant from three and six day harvests

5.6 Sequences of light chains expressed

Amino acid sequences of the six hybrid light chains and two point mutations of B3V_L are compared with wild-type IS4V_L, UK4V_L, B3V_L and germline gene 2a2, in Figure 5.11 A). Sequence characteristics of the wild-type V_L have been discussed previously (chapter 3.4 and 3.5) and all of the mutants and hybrids are derived from one or more of these light chains.

The mutant form of B3V_L shown in Figure 5.11 was generated by Dr A. Rahman and cloned into expression vectors by Dr J. Haley (Rahman et al., 2001). This mutant (named

$B3V_La$) contains an Arg residue at position 27a in CDR1 reverted to Ser, the amino acid found at this position in the germline gene 2a2.

Figure 5.11 also shows the amino acid sequences of the V_L CDR hybrids in which each newly formed chain construct contains CDR1 of one antibody with CDR2 and CDR3 of a different antibody.

5.7 Expression of whole IgG molecules in COS-7 cells

Each of the 10 light chains shown in Figure 5.11 A) was paired with $B3V_H$ and $IS4V_H$. Each of the six mutant forms of $IS4V_H$, see Figure 5.11 B) and legend to Table 2.7 (page 153), was paired with $IS4V_L$ and $B3V_L$. A total of 32 heavy/light chain combinations were expressed in COS-7 cells. At least two expression experiments were carried out for each combination. IgG was obtained in the supernatant for all of the combinations. The concentrations of IgG in COS-7 cell supernatants from each of the 32 heavy/light chain combinations are shown in Table 5.3. In each case the negative control sample, in which COS-7 cells were electroporated without any plasmid DNA, contained no detectable IgG. Consistently high yields were obtained with the $B3V_H/BIV_L$, $B3V_H/UIV_L$ and $IS4V_H/UIV_L$ combinations compared to the other antibody combinations.

5.8 Results of aCL ELISA

For each heavy/light chain combination, which bound CL, the linear portion of the binding curve for absorbance against antibody concentration was determined empirically by dilution of antibody over a range of concentrations. Similar patterns of binding were obtained with each antibody combination from repeated expression experiments and representative results from two experiments (A and B) are shown for Figures 5.12 to 5.16. A heavy/light chain combination was not considered to bind CL if its binding curve gave an absorbance of less than 0.1 (at 405nm) for all concentrations tested in the aCL ELISA.

A) Lambda chains

```
      FR1                       CDR1           FR2              CDR2    FR3                                CDR3     Jλ
      1                   20    24 27          34 35   40       50         60        70        80          89            100
      |                   |     |  | abc       |  |    |        |          |         |         |           |             |
2a2   QSALTQPASVSG.SPGQSITISC TGTSSDVGGYNYVS WYQQHPGKAPKLMIY EVSNRPS GVSNRFSGSKSGNTASLTISGLQAEDEADYYC SSYTSSST VVFGGGTKLTVLG
IS4   ------------.---------- --A---I-S--S-- ---HL-------I-- D--S--- ------------F---------P-------- C---TIN- W------------
UK4   ------------.---------- ---SN---S----- ---L---E----L-- DAIK--- ---------E-----------------G---- ----NR.. -F-----------
B3    ------------.---------- ---RR------F-- --------------- ---H--- ---T---A----S------------------- ---S-TTR -------------
B3a   ------------.---------- ---R-------F-- --------------- ---H--- ---T---A----S------------------- ---S-TTR -------------
IB    ------------.---------- --A---I-S--S-- --------------- ---H--- ---T---A----S------------------- ---S-TTR -------------
IU    ------------.---------- --A---I-S--S-- ---L---E----L-- DAIK--- ---------E-----------------G---- ----NR.. -F-----------
UI    ------------.---------- ---SN---S----- ---HL-------I-- D--S--- ------------F---------P-------- C---TIN- W------------
UB    ------------.---------- ---SN---S----- --------------- ---H--- ---T---A----S------------------- ---S-TTR -------------
BI    ------------.---------- ---RR------F-- ---HL-------I-- D--S--- ------------F---------P-------- C---TIN- W------------
BU    ------------.---------- ---RR------F-- ---L---E----L-- DAIK--- ---------E-----------------G---- ----NR.. -F-----------
```

B) Heavy chains

```
      FR1                               CDR1  FR2            CDR2              FR3                              CDR3             JH
                                     30 31    36   40        50           60     70           82      90             100         101
                                      | |     |    |         |            |      |            | abc   |              |           |
1-03  QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYAMH WVRQAPGQRLEWMG WINSGNGNTKYSQKFQG RVTITRDTSASTAYMELSSLRSEDTAVYYCAR                 YFDYWGQGTLVTVSS
IS4   ---F--------------------F--S-- --FL- --------GP---- -------------N--- --S--------VV-------G----------- GRRDVRGVLWRGRHD ---F-------I---

3-23  EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVS AISGSGGSTYYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK                 YFDYWGQGTLVTVSS
B3    ----V------------------T-----T T---- -------------- T---R-S-----G--Q- ---L------S--------------------- PNVGSGW         S--S-----L-T---
```

Figure 5.11 Sequence alignment of expressed V_L and V_H regions

Sequences were aligned using DNAplot VBASE (Tomlinson *et al.*, 1996). A) Sequences of expressed V_λ regions compared to 2a2. B) Sequences of expressed V_H regions compared to 1-03 (IS4), 3-23 (B3). The D_H regions could not be matched to germline genes. Arginine residues altered by site directed mutagenesis to serine residues in IS4V_H CDR3 are underlined. The amino acids are numbered according to Kabat. Dots have been inserted to facilitate the alignment. A dash indicates homology with the corresponding germline sequence. Abbreviations:- FR – Framework region; CDR – Complementarity determining region.

Heavy chain	Light chain	IgG Concentration (ng/ml)	CL Binding
IS4	IS4	24-368	+
IS4	IB	22-140	+
IS4	IU	70-194	-
IS4	B3	5-14	++
IS4	BI	50-60	++
IS4	BU	5-60	-
IS4	UK4	11-22	-
IS4	UI	50-480	+
IS4	UB	9-50	-
B3	IS4	71-192	-
B3	IB	41-96	-
B3	IU	89-376	-
B3	B3	6	+
B3	BI	120-608	-
B3	BU	40-68	-
B3	UK4	8-28	-
B3	UI	60-480	-
B3	UB	2-20	-
IS4	B3a	48-60	++
B3	B3a	2.5-4	-
IS4V_Hi	IS4	50-56	-
IS4$V_H$ii	IS4	65-70	-
IS4$V_H$iii	IS4	48-90	-
IS4$V_H$iv	IS4	48-90	-
IS4V_Hx	IS4	78-94	-
IS4V_Hi&ii	IS4	74-80	-
IS4V_Hi	B3	24-54	++
IS4$V_H$ii	B3	30	++
IS4$V_H$iii	B3	30-34	+
IS4$V_H$iv	B3	28-30	+
IS4V_Hx	B3	32-34	-
IS4V_Hi&ii	B3	32-47	++

Table 5.3 Range of IgG concentrations in ng/ml produced by expression of the heavy/light chain combinations and a summary of their ability to bind CL

For a description of B3a and IS4V_H mutants see section 5.6 and legend to Table 2.7, page 153, respectively.

5.9 The importance of arginine residues in IS4V_H

As shown in chapter four, the presence of the heavy chain of IS4 plays a dominant role in binding to CL (Giles et al., 2003). IS4V_H binds CL in combination with five of the nine light chains tested (Figures 5.12 A and B). These were B3V_L, BIV_L, IS4V_L, IBV_L and UIV_L, but only one of these light chains (B3V_L) binds CL in combination with B3V_H (Figures 5.13 A and B). Despite being tested over a wide range of antibody concentrations, none of the other combinations showed any binding to CL at all.

To identify the features of IS4V_H that show enhanced binding to CL, I focused on the combination IS4V_H/B3V_L. This combination shows high binding to CL. This binding could be altered by the replacement of some or all of the four surface-exposed Arg in IS4V_H CDR3 to Ser, as shown in Figures 5.14 A) and B). Substitution of all four Arg with Ser residues (IS4V_Hx) abolished CL binding completely. This effect seems likely to be due entirely to the changes at positions 100 and 100g. This is supported by the fact that heavy chain combinations containing Arg to Ser mutations at these positions (IS4$V_H$iii and IS4$V_H$iv) displayed reduced binding to CL as shown by the downward shift of the curve compared to the wild-type IS4V_H/B3V_L combination. In contrast, there were no reductions in CL binding for the heavy chains containing Arg to Ser mutations at position 96 (IS4V_Hi), position 97 (IS4$V_H$ii) or both (IS4V_Hi&ii).

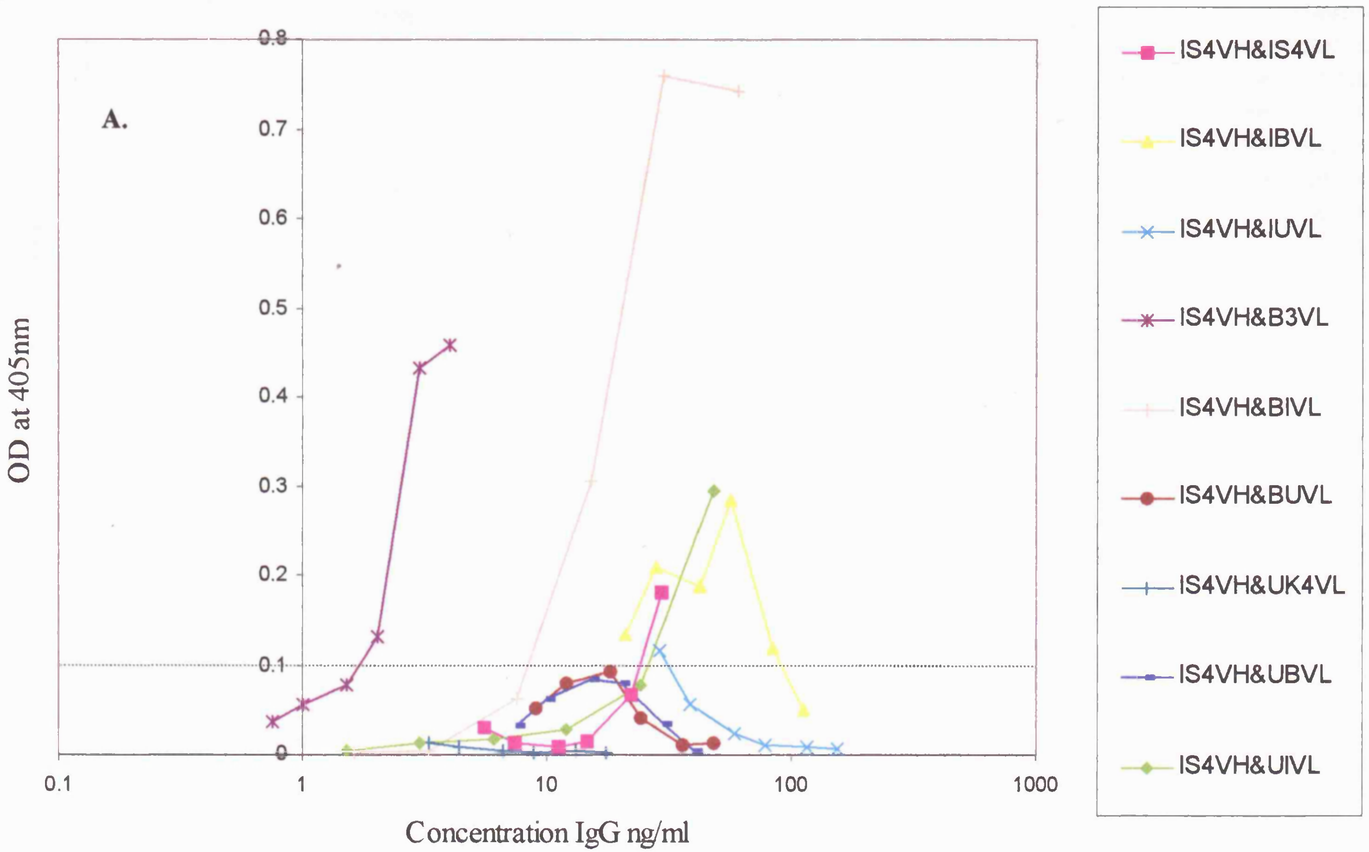


Figure 5.12 A) Effect of CDR exchange in the light chains

Cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type IS4V_H expressed with wild-type or hybrid light chains

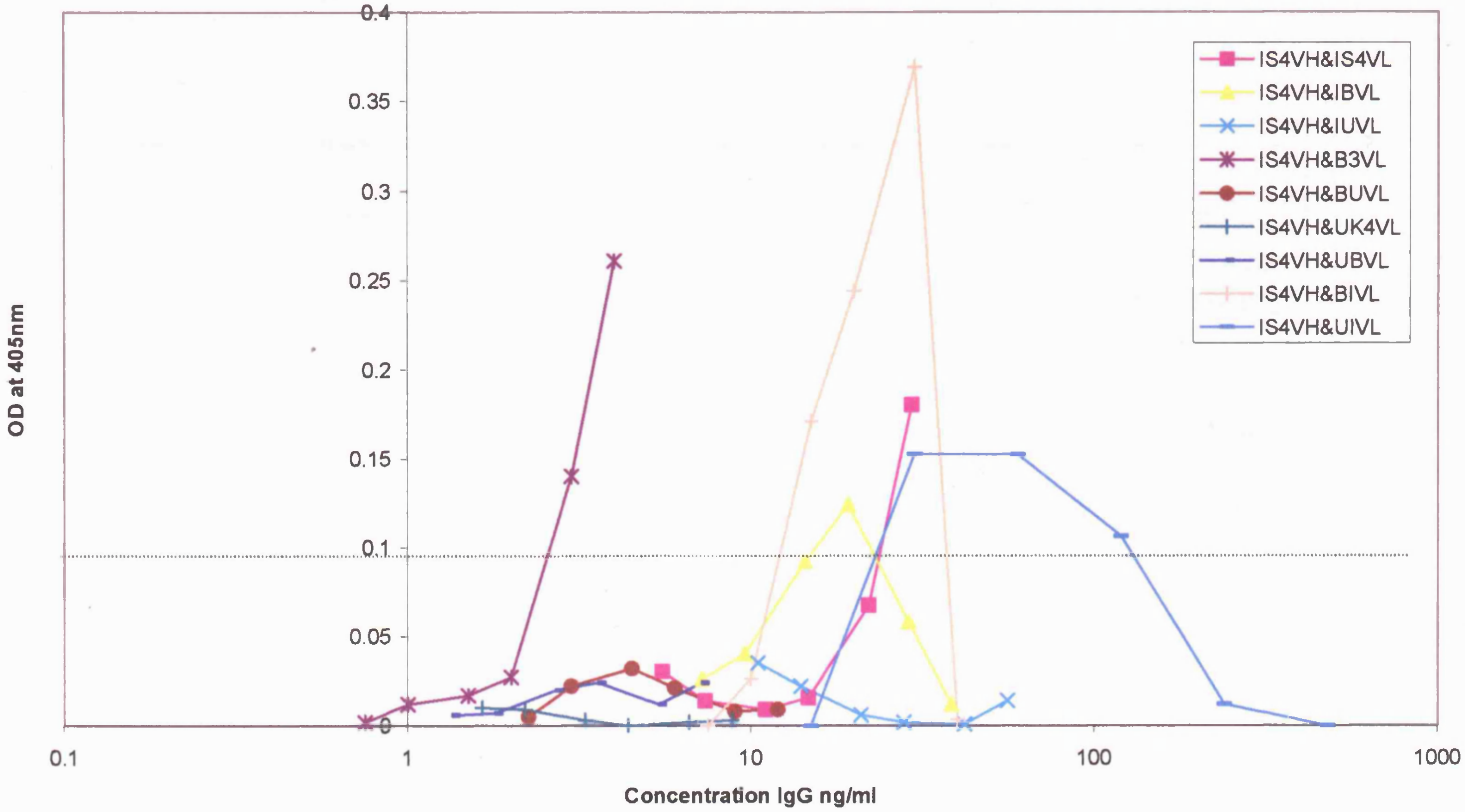


Figure 5.12 B) Effect of CDR exchange in the light chains

For legend see Figure 5.12 A).

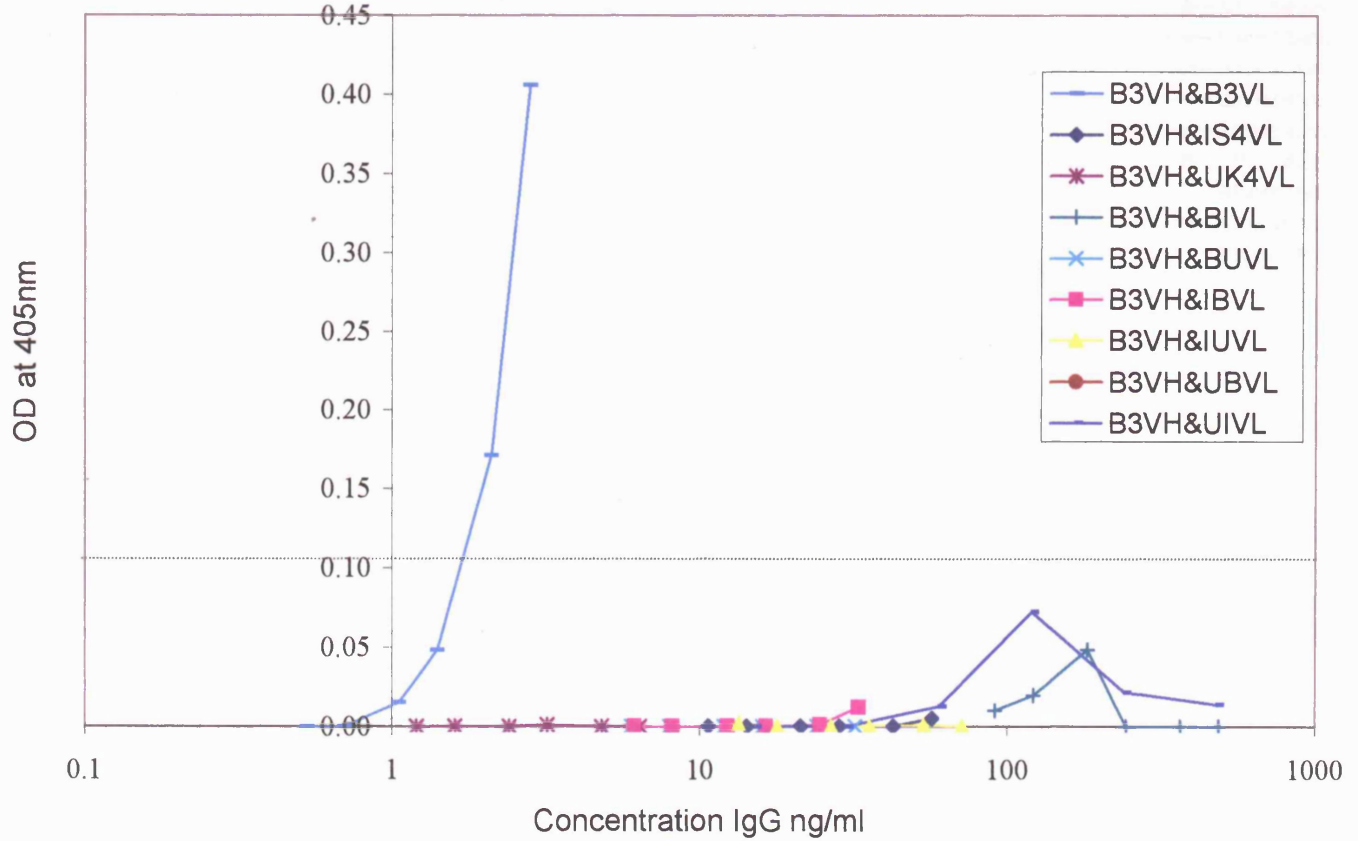


Figure 5.13 A) Effect of CDR exchange in the light chains

Cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type B3V_H expressed with wild-type or hybrid light chains

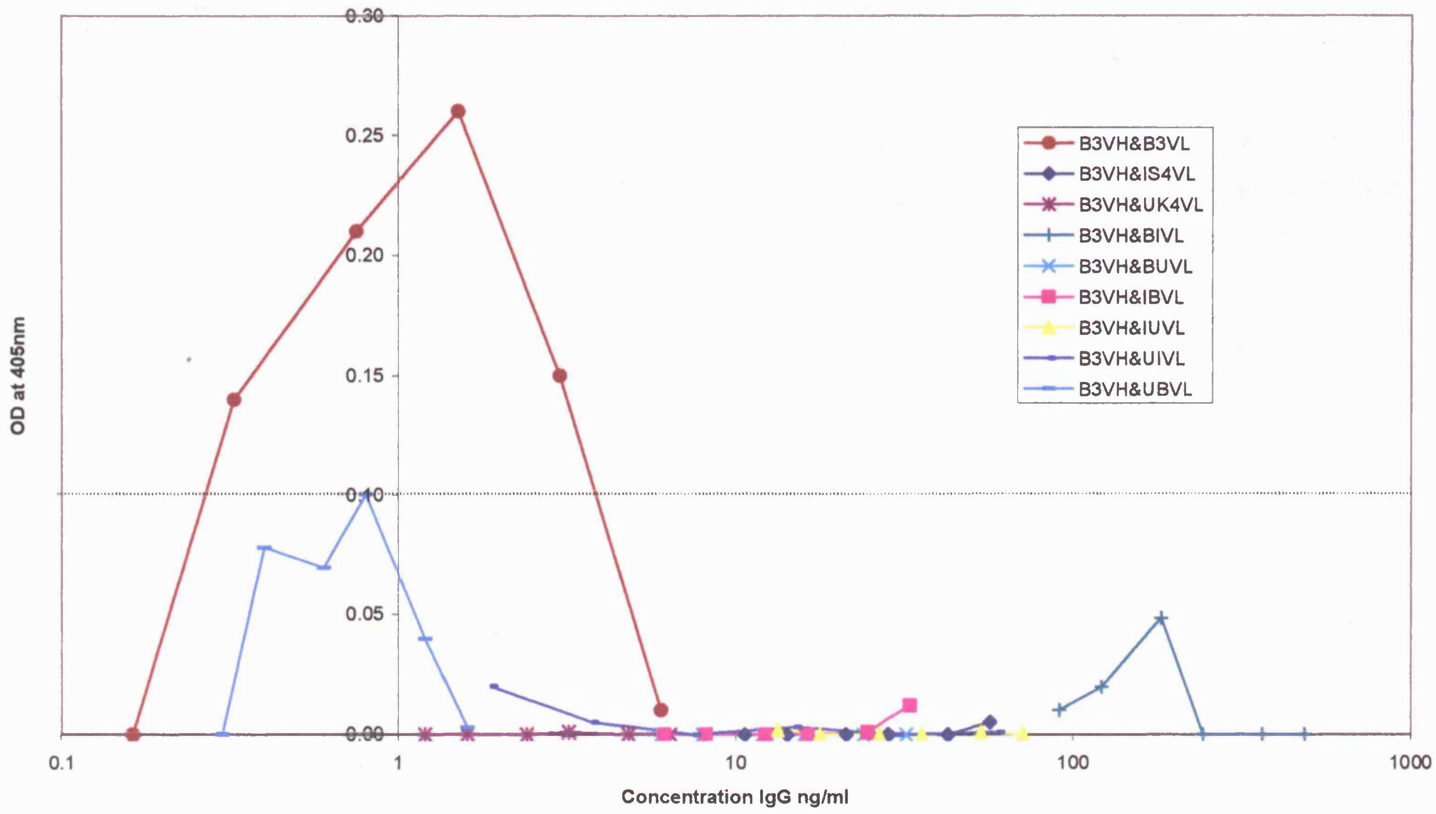


Figure 5.13B) Effect of CDR exchange in the light chains

For legend see Figure 5.13 A).

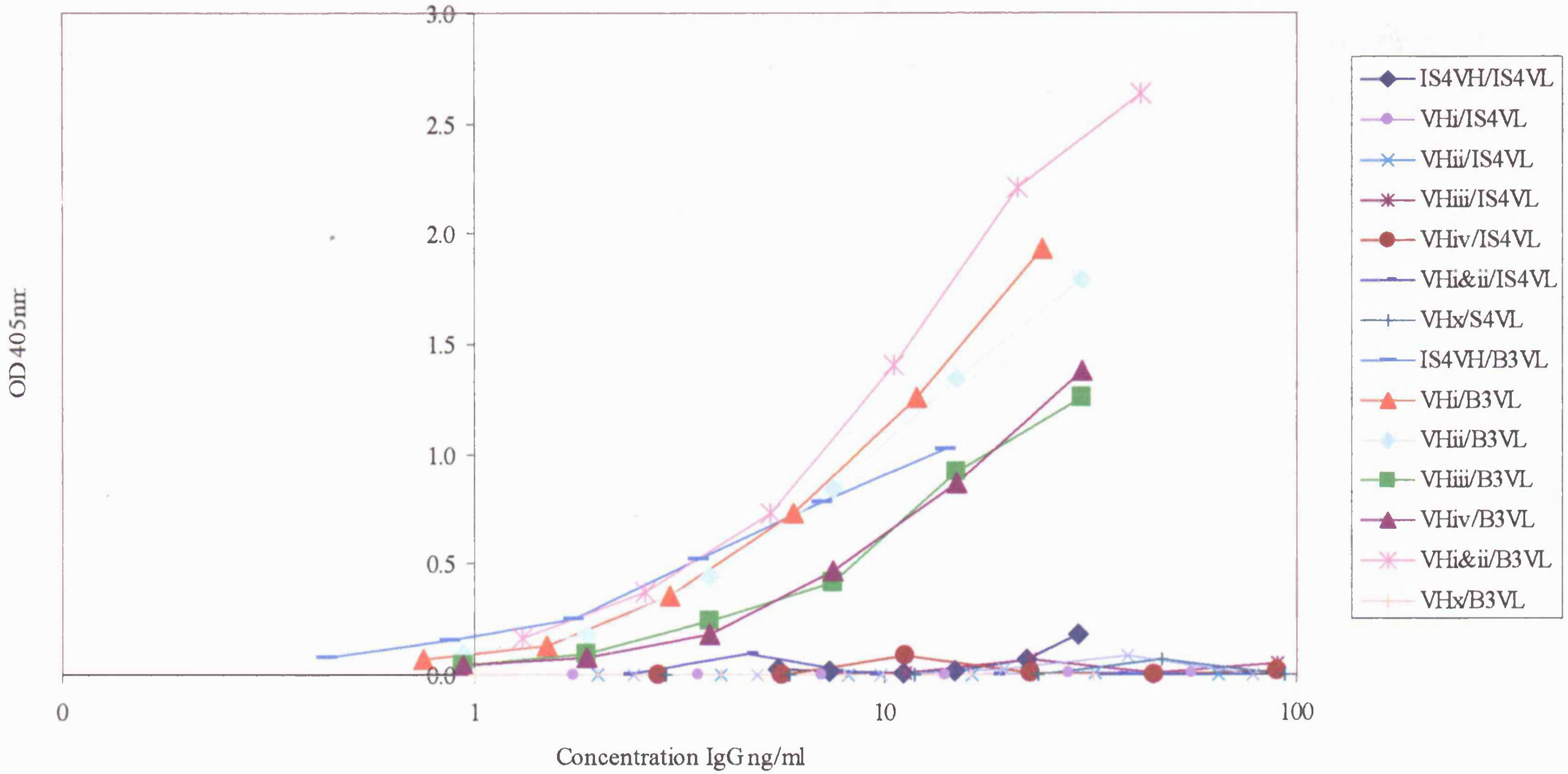


Figure 5.14 A) Effect of Arg to Ser point mutations in IS4V_HCDR3
Cardiolipin binding of IgG in COS-7 cell supernatants containing wild type or mutant forms of IS4 heavy chain expressed with wild type B3 or IS4 light chains. The IS4V_H mutants VHi, VHii, VHiii and VHiv contain single Arg to Ser point mutations at positions 96, 97, 100 and 100g respectively; whilst VHi&ii contains Arg to Ser at positions 96 and 97; and VHx has an Arg to Ser mutation at all four positions.

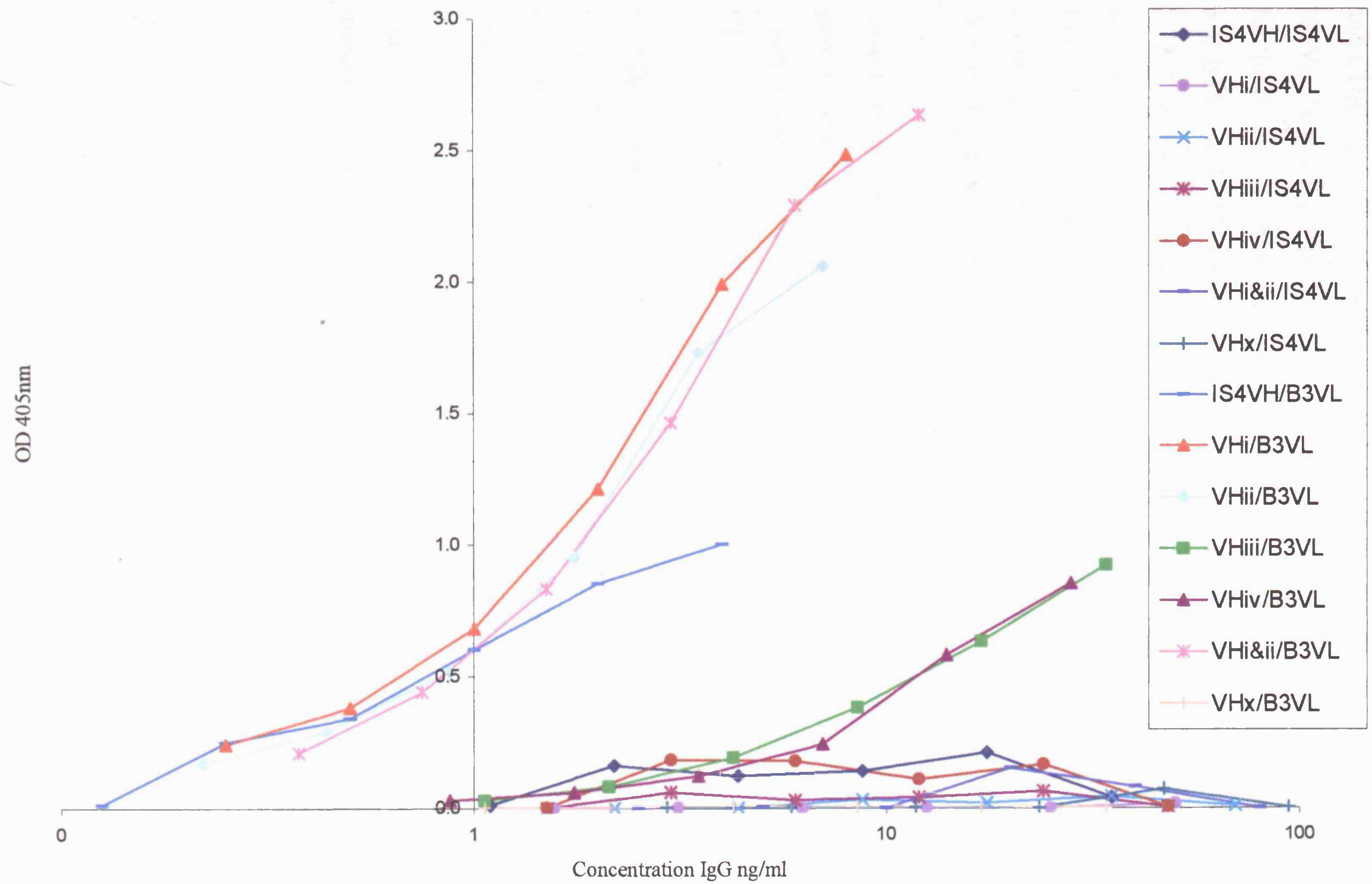


Figure 5.14 B) Effect of Arg to Ser point mutations in IS4V_HCDR3

For legend see Figure 5.14 A).

5.10 The importance of arginine residues in the light chain CDRs

Five light chains bound CL in conjunction with $IS4V_H$; see Figures 5.12 A) and B). The strongest binding was seen with light chains containing $B3V_LCDR1$, namely $B3V_L$ and BIV_L, in combination with $IS4V_H$. In contrast, light chains IB and UB, containing CDR2 and CDR3 from B3, showed weak and no binding to CL respectively in combination with $IS4V_H$. To test the hypothesis that the arginine at position 27a in $B3V_LCDR1$ is responsible for the favourable effect of this CDR on binding to CL, I expressed combinations of $IS4V_H$ and $B3V_H$ with $B3V_La$, in which Arg 27a has been mutated to Ser. As shown in Figures 5.15 A) and B), there was a significant decrease in CL binding of $B3V_H/B3V_La$ compared with $B3V_H/B3V_L$. Although the combination $IS4V_H/B3V_La$ binds CL less strongly than $IS4V_H/B3V_L$, reduction in binding is not as great as that seen when these light chains are combined with B3VH. This observation is consistent with the idea that $IS4V_H$ plays a dominant role in binding to CL.

Despite being tested at a range of concentrations up to 75 times higher than those which gave maximal CL binding for the other combinations containing $IS4V_H$ none of the light chains containing $UK4V_L$ CDR2 and 3 (UK4 wild type, IU and BU) showed any binding to CL.

5.11 Results of anti-dsDNA ELISA

Figures 5.16 A) and B) shows anti-DNA binding of combinations of $IS4V_H$ or $B3V_H$ with each of the wild type or hybrid light chains. Only the combination $B3V_H/B3V_L$ showed significantly more binding than any other combination. Anti-dsDNA ELISAs were not performed upon the V_H/V_L combinations containing point mutations in their heavy or light chains, since $IS4V_H$ does not confer the ability to bind DNA and the effects of light chain mutations in $B3V_L$ have already been well established (Rahman et al., 2001).

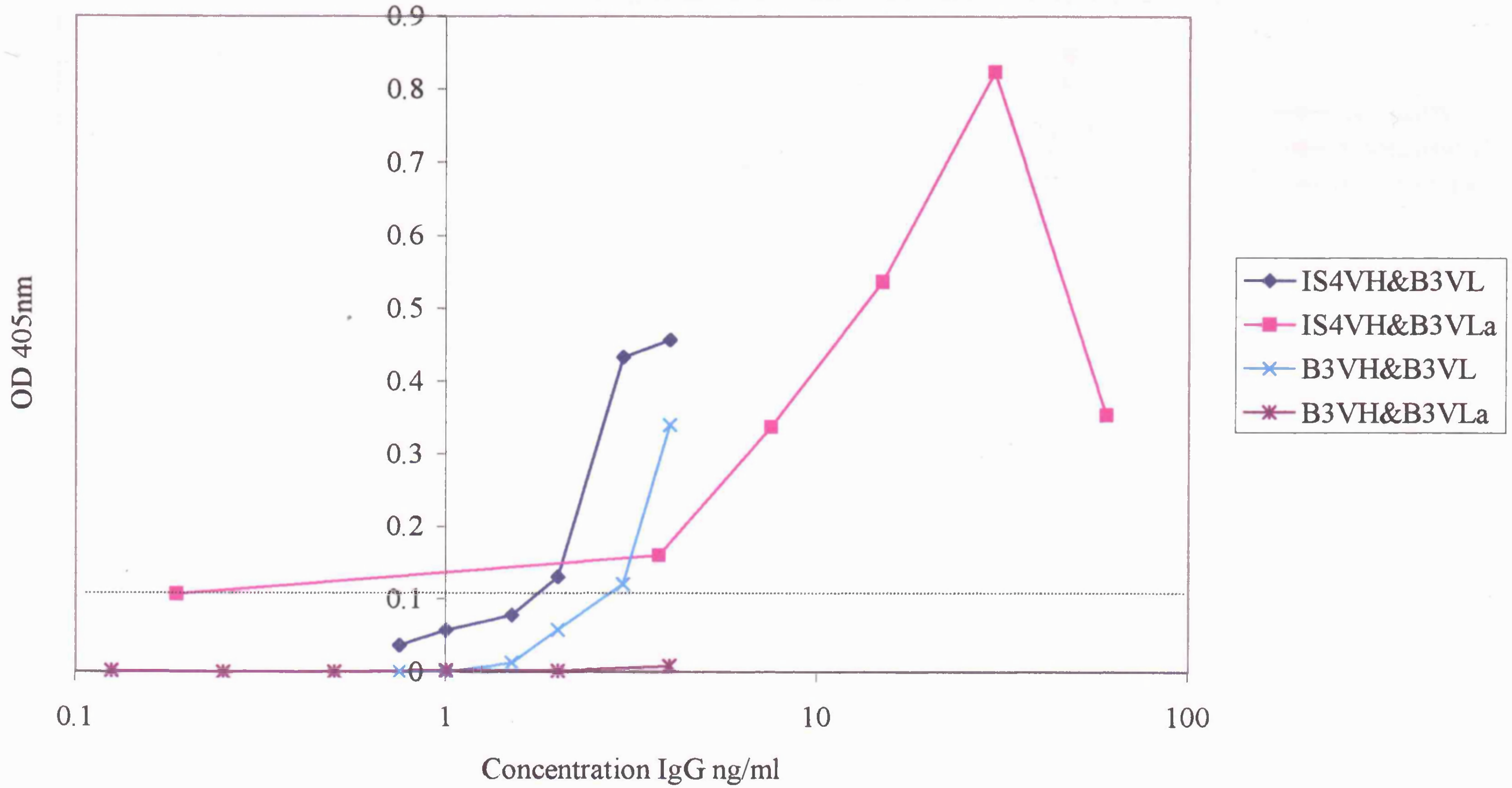


Figure 5.15 A) Effect of point mutation Arg 27a to Ser in B3 V_LCDR1

Comparison of cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type heavy chains expressed with B3V_L or B3V_La.

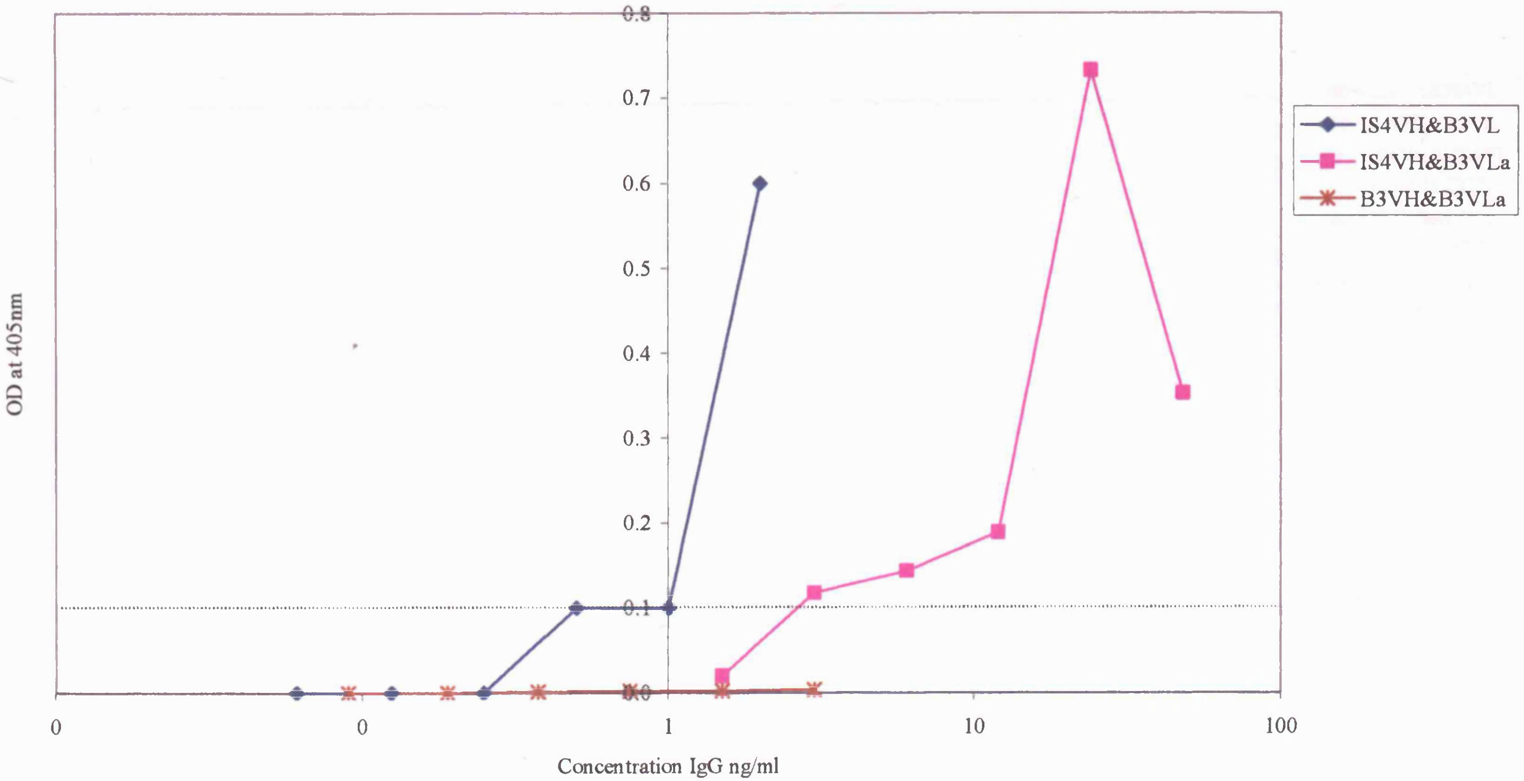


Figure 5.15 B) Effect of point mutation Arg 27a to Ser in B3 V_LCDR1

Comparison of cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type heavy chains expressed with B3V_L or B3V_La. The B3V_H/B3V_L combination did not produce any IgG on this occasion therefore is not shown.

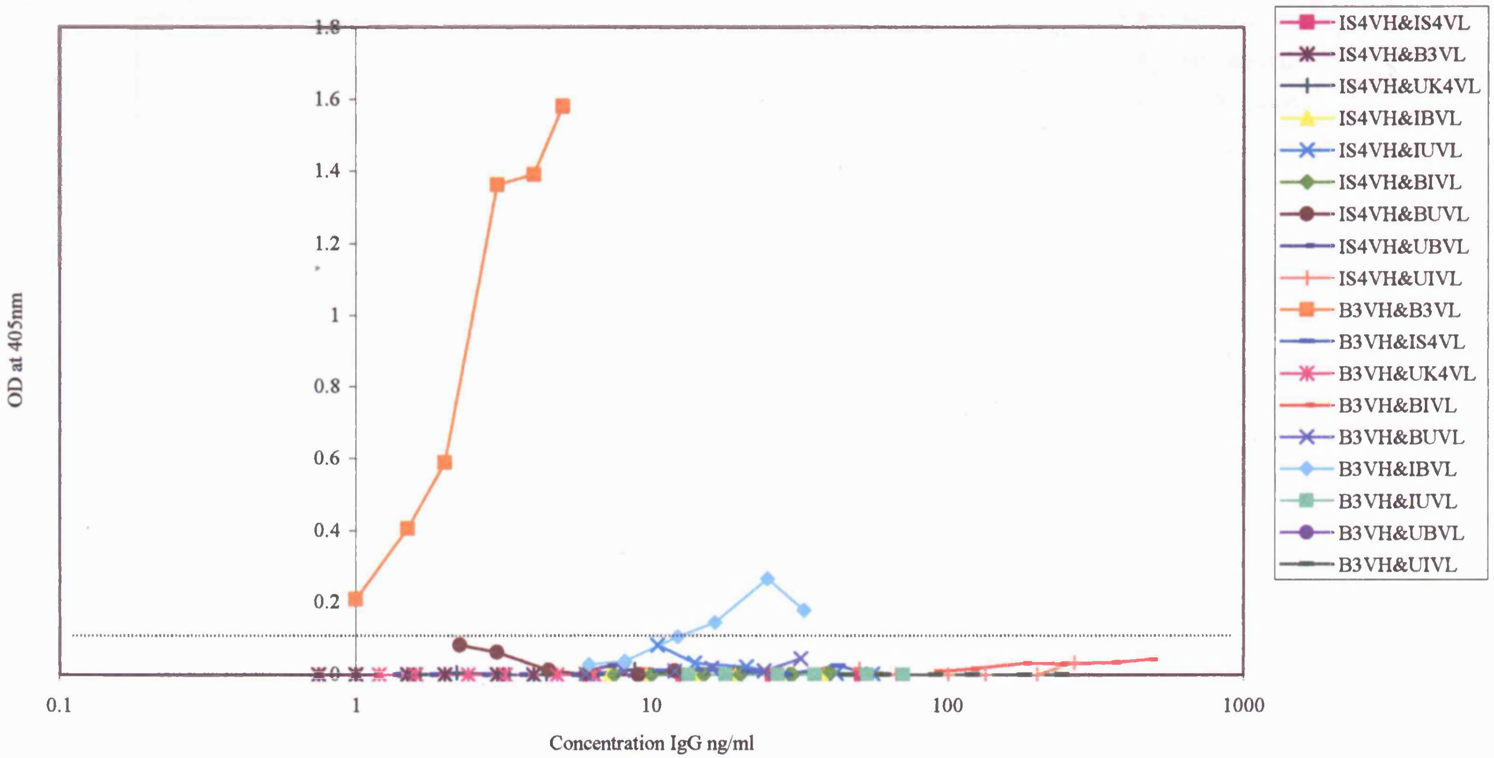


Figure 5.16 A) Results of anti-dsDNA ELISA

The graph shows binding of IgG in COS-7 cell supernatants containing each heavy light chain combination to dsDNA.

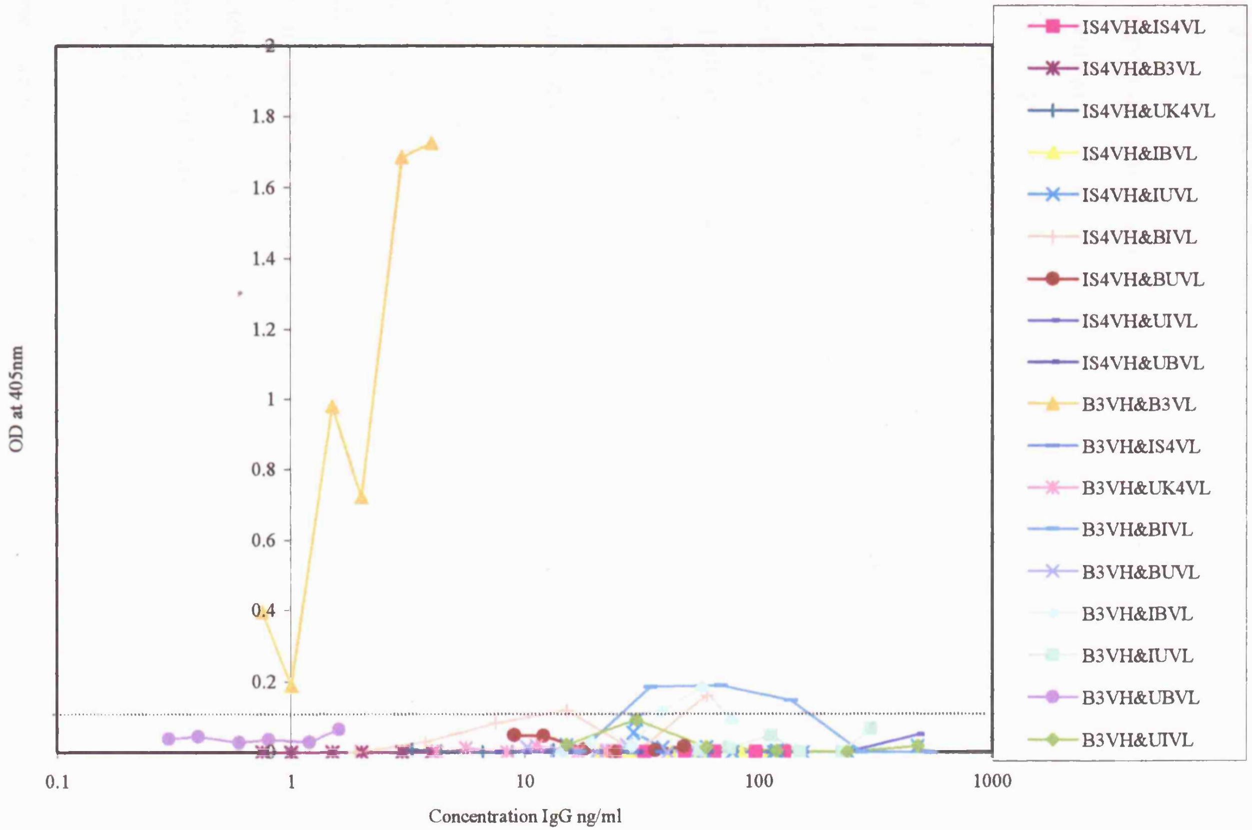


Figure 5.16 B) Results of anti-dsDNA ELISA

For legend see Figure 5.16 A)

5.12 Discussion of results

Based on the results of sequence analysis, modelling studies (see chapters 3 and 4) and heavy/light chain exchange experiments (in chapter 4) I postulated that surface exposed Arg residues in IS4V_H and B3V_L may be important for CL binding. The results described in this chapter complement my earlier findings and support my hypothesis as well as showing that it is not just the presence but the precise location of Arg residues in the CDRs that is important in determining ability to bind CL.

The importance of Arg residues at specific positions in the V_H and V_L sequences of anti-DNA antibodies has been examined by many groups by expressing the antibodies *in-vitro*, and then altering the sequence of the expressed Ig by chain swapping or mutagenesis. In general, these studies have shown that altering the numbers of Arg residues in the CDRs of these antibodies can lead to significant alterations in binding to DNA. Arg in V_HCDR3 often play a particularly important role in binding to this antigen (Radic *et al.*, 1991; Mockridge *et al.*, 1996; Pewzner-Jung *et al.*, 1996; Li *et al.*, 2000; Rahman *et al.*, 2001). Recently it was shown that the affinity of human phage derived anti-dsDNA Fabs from a lupus patient correlated with the presence of somatically mutated Arg residues in CDR1 and CDR2 of the heavy chains (Behrendt et al., 2003).

Previous studies of the contribution of aPL heavy or light chains to CL binding have yielded conflicting results. Different groups have reported important contributions from the heavy chain (Blank et al., 1999; Cocca et al., 2001), the light chain (Pereira et al., 1998), or both (Pewzner-Jung *et al.*, 1996; Kumar *et al.*, 2000). In one of these studies the role of Arg residues was examined in a murine antibody (3H9) with dual specificity for PL and DNA. The introduction of Arg residues into the heavy chain V region at positions known to mediate DNA binding enhanced binding to PS-ß$_2$GPI complexes and to apoptotic cell debris, which may be an important physiological source of both these antigens (Cocca et al., 2002).

These data shows that combinations of IS4V_H with light chains containing CDR1 of B3 (B3 wild type and BI) produced the strongest binding to CL. This CDR contains two surface exposed Arg residues at positions 27 and 27a. Previous modelling studies have suggested that the binding of B3V_H/B3V_L to dsDNA is stabilised by the interaction of dsDNA with

Arg 27a in CDR1 and Arg 54 in CDR2 of the light chain (Kalsi et al., 1996). Expression and mutagenesis studies from our group confirmed that mutation of Arg27a to Ser led to a reduction in binding to DNA (Rahman et al., 2001). In this paper the same change has been shown to reduce binding to CL, supporting the conclusion of Cocca *et al.* (Cocca et al., 2001) that Arg at particular positions can enhance binding to both DNA and CL.

The presence of UK4V_L CDR 2 and 3 in any light chain blocked binding to CL, even when combined with B3V_LCDR1 (light chain BU). UK4V_LCDR1, however, does not block binding. Our group has previously shown that the presence of UK4V_LCDR2 and 3 blocks binding to DNA and histones but not to the Ro antigen (Rahman *et al.*, 2001; Haley *et al.*, 2003). Modelling studies have shown that an Arg at position 94 in CDR3 of UK4V_L hinders DNA binding sterically. A similar effect may be occurring with regards to the binding of UK4V_L to CL.

The effect of point mutations of specific Arg residues in CDR3 of IS4V_H upon CL binding is shown in Figures 5.14 A) and B). The low binding of IS4V_H/IS4V_L was abolished by inclusion of any one of these mutations. This is not the case, however, when these mutants are expressed with B3V_L. In this case the Arg residues at 100 and 100g confer a greater effect on CL binding compared to the Arg residues at positions 96 and 97. Substitutions of all four of these IS4V_H CDR3 Arg residues were sufficient to completely abolish all binding to CL.

These data confirm that the effect of Arg residues on binding to CL is highly dependent on the positions that they occupy in the sequence. The precise location of Arg residues has been shown to be important in the binding of both murine and human anti-dsDNA to DNA in numerous studies (Radic et al., 1993; Radic and Weigert, 1994; Rahman et al., 2001). Interestingly, Krishnan *et al.* (Krishnan et al., 1996; Krishnan and Marion, 1998) have demonstrated a strong correlation between specificity for dsDNA and the relative position of Arg residues in V_HCDR3. They reported that the frequency of Arg expression among murine anti-dsDNA binding Abs was highest at position 100 and postulate that the importance of this residue in binding to dsDNA lies in its position at the centre of the V_HCDR3 loop in the structure of the antigen combining site (Krishnan et al., 1996).

Assuming that this loop would be projected outward from the antigen-combining site, an Arg at position 100 would be located at the apex of the V_HCDR3 loop.

In summary, I have demonstrated the relative importance of certain surface exposed Arg residues at critical positions within the light chain CDR1 and heavy chain CDR3 of different human mAbs in conferring the ability to bind CL in a direct ELISA. It is now important to test the effects of sequence changes involving these amino acids on pathogenic functions of these aPL, by expressing the altered antibodies in larger quantities from stably transfected (CHO) cells. More accurate information about changes in affinity of variant forms of aPL for binding to CL or β_2GPI could be obtained by testing larger quantities of purified antibodies in assays such as SPR or inhibition ELISA. Pathogenic properties of these variant forms of aPL could then be tested both in *in-vivo* and *in-vitro* models of the APS.

CHAPTER 6

STABLE EXPRESSION OF WHOLE IgG FROM CLONED ANTIBODY cDNA IN CHO*dhfr*⁻ CELLS

6.1 *Eco*R I digest of pG1D210 containing IS4V_H and light chain expression vector

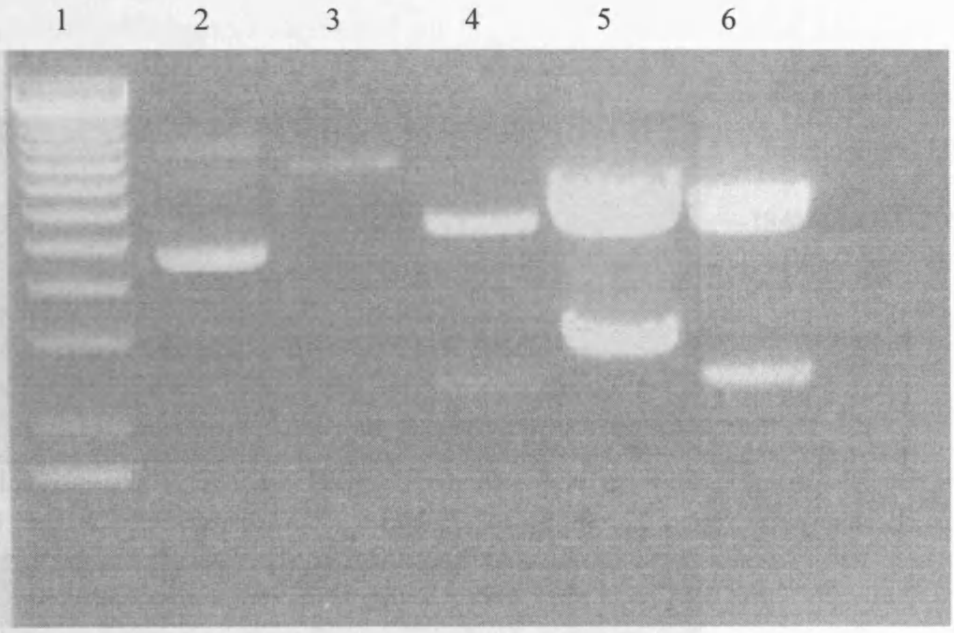
The constituent parts (and *Eco*R I fragment size) of each supervector are shown in Table 6.1. The results of an *Eco*R I digest are shown in Figure 6.1. Bands of the appropriate size (see Table 6.1) were cut out and purified (as in chapter 2.8) for subsequent ligation.

HEAVY CHAIN (*Eco*R I fragment size)	LIGHT CHAIN (Parent plasmid, *Eco*R I fragment size)	SUPERVECTOR (Overall size)
IS4 (7674)	IS4 (pLN100, 2670)	SVIS4V_H/IS4V_L (10344)
IS4 (7674)	B3 (pLN10, 2310)	SVIS4V_H/B3V_L (9984)
IS4 (7674)	UK4 (pLN10, 2304)	SVIS4V_H/UK4V_L (9978)

Table 6.1 Components of each supervector

6.2 Large scale screening of colonies from supervector ligations

The rapid screening of large numbers of colonies was possible using the osmotic lysis technique described in section 2.20.4. In Figure 6.2 A), 18 colonies (lanes 2-19) from two LB/Agar/Amp plates containing different insert:vector ratios of IS4V_H and UK4V_L and two colonies (lanes 1 and 20) containing re-ligated vector only in the absence of insert, have been screened. The only positive clone in this instance occurred in lane 10 where a "step" is clearly visible indicating an increase in size of the plasmid due to the successful ligation of UK4V_L into IS4V_H/pG1D210. Figure 6.2 B) shows the result from screening of 18 colonies from a single LB/Agar/Amp plate containing IS4V_H (vector) and IS4V_L (insert) and two control colonies (lanes 1 and 20) containing vector only. In this case there were four positive clones in lanes 4, 10, 11 and 19. These clones of potentially the right size were selected and the remaining 100μl aliquot of culture was used to inoculate broth for purification and further characterization of the plasmids.



Lanes
1 = 1Kb marker
2 = Uncut IS4V_H/pG1D210
3 = *Eco*R I digested IS4V_H/pG1D210
4 = *Eco*R I digested UK4V_L/pLN10
5 = *Eco*R I digested IS4V_L/pLN100
6 = *Eco*R I digested B3V_L/pLN10

Figure 6.1 Preparation of *Eco*R I fragments for creation of supervectors

*Eco*R I digest of: - IS4V_H/pG1D210 (7674bp) in lane 3; UK4V_L/pLN10 (5187bp and 2304bp) in lane 4; IS4V_L/pLN100 (5187bp and 2670bp) in lane 5; and B3V_L/pLN10 (5187bp and 2310bp) in lane 6. Uncut IS4V_H/pG1D210 is shown for comparison in lane 2. The smaller fragments in lanes 4 – 6 contain the light chain variable region.

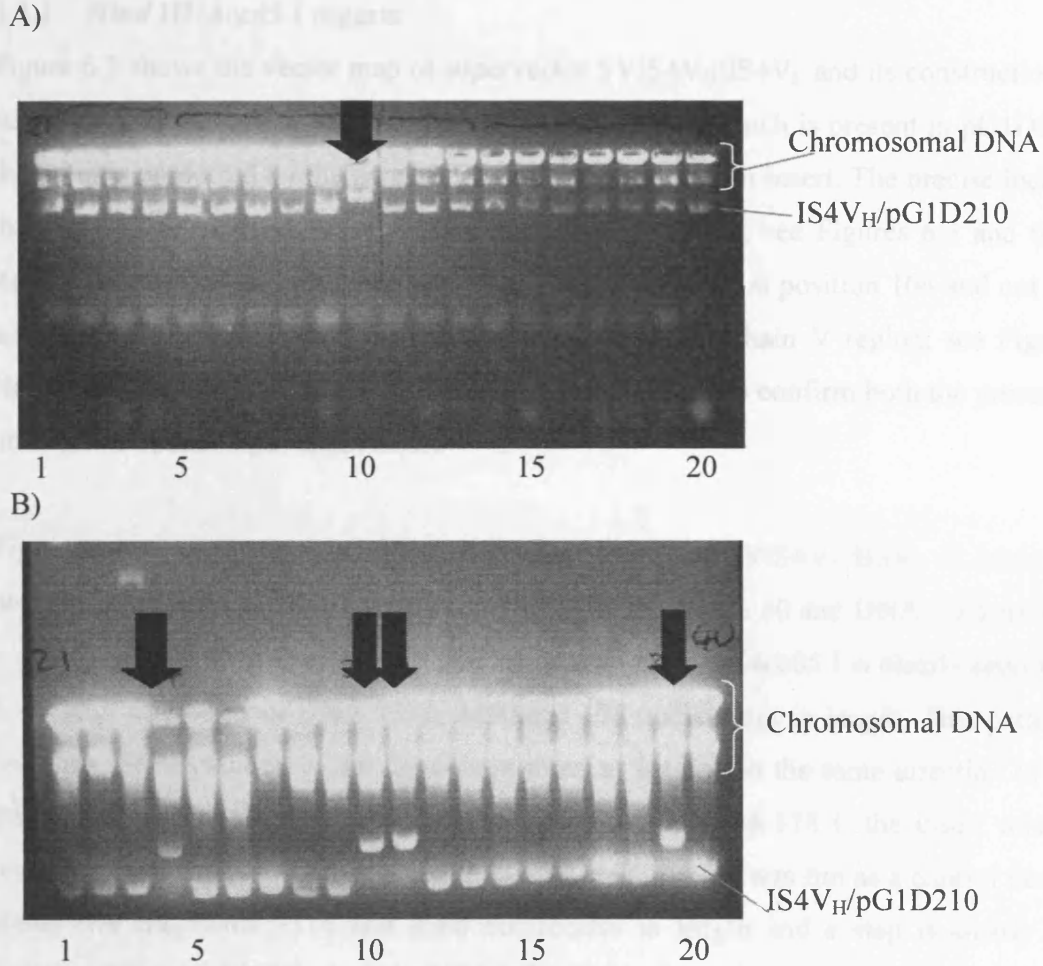


Figure 6.2 Large scale screening of supervectors

A) Colonies from a ligation of an *Eco*R I cassette containing UK4V_L into *Eco*R I digested IS4V_H/pG1D210 which is 7674bp long. In lane 10 (arrowed) the presence of a supervector is suggested by the presence of a larger band of DNA which should be 9978bp long for SVIS4V_H/UK4V_L.

B) Similarly colonies from a ligation of an *Eco*R I cassette containing IS4V_L into *Eco*R I digested IS4V_H/pG1D210. Lanes 1 and 20 contain the transformation control of uncut IS4V_H/pG1D210 which is 7674bp long. In lanes 4, 10, 11 and 19 (arrowed) the presence of a supervector is suggested by the presence of a larger band of DNA which should be 10344bp long for SVIS4V_H/IS4V_L.

6.3 Confirmation restriction digests of supervectors

6.3.1 *Hind* III/*Acc65* I digests

Figure 6.3 shows the vector map of supervector SVIS4V_H/IS4V_L and its construction. Each supervector contains two *Hind* III restriction sites one of which is present in pG1D210 and the other is conferred by the parent plasmid of the light chain insert. The precise location of the *Hind* III site differs slightly between each supervector, see Figures 6.3 and 6.4. The *Acc*65 I site however is unique to each light chain V region at position 106 and not present anywhere else in either of the parent plasmids or heavy chain V region, see Figure 6.3. Hence the *Hind* III and *Acc*65 I restriction sites were ideal to confirm both the presence and orientation of each light chain insert.

Figure 6.4 A) shows a *Hind* III/*Acc*65 I dual digest of SVIS4V_H/B3V_L in lanes 2 – 9, compared to SVIS4V_H/B3V_L single cut with *Hind* III in lane 10 and DNA markers in lanes 1 and 11. The expected pattern of three bands with *Hind* III/*Acc*65 I is clearly seen in lanes 3 - 8 with fragments of DNA 7316, 2490 and 178 nucleotides in length. This pattern also confirms the orientation of the light chain insert as reading in the same direction as the V_H insert because the fragment sizes would be 7000, 2806 and 178 if the insert was in the reverse orientation. The single *Hind* III cut SVIS4V_H/B3V_L was run as a control because it yields two fragments 7316 and 2668 nucleotides in length and a step is clearly visible distinguishing the 2490 fragment of SVIS4V_H/B3V_L (lanes 3 – 8) and the 2668 fragment in lane 11, when the samples (seen in 6.4 A) were run overnight at 10 Volts, see 6.4 B).

A similar pattern is seen in Figure 6.4 C) which displays the Hind III/Acc65 I dual digest of SVIS4V_H/UK4V_L in lanes 3 – 7 and SVIS4V_H/IS4V_L in lanes 8 – 11 compared to SVIS4V_H/B3V_L single cut with *Hind* III in lane 12 and DNA markers in lanes 1 and 2. The fragment sizes seen of these two supervectors are those expected with the light chain inserts in the correct orientation and are 7316, 2484 and 178 for SVIS4V_H/UK4V_L; and 7676, 2490 and 178 for SVIS4V_H/IS4V_L. If the inserts were in the reverse orientation then sizes of 6994, 2806 and 178 for SVIS4V_H/UK4V_L and 7100, 3166 and 178 for SVIS4V_H/IS4V_L would have been obtained. A step is once again visible between the 2668 fragment in lane 12 and all of the middle fragments in lanes 3 – 11 and 13, which are smaller in size hence confirming their correct orientation.

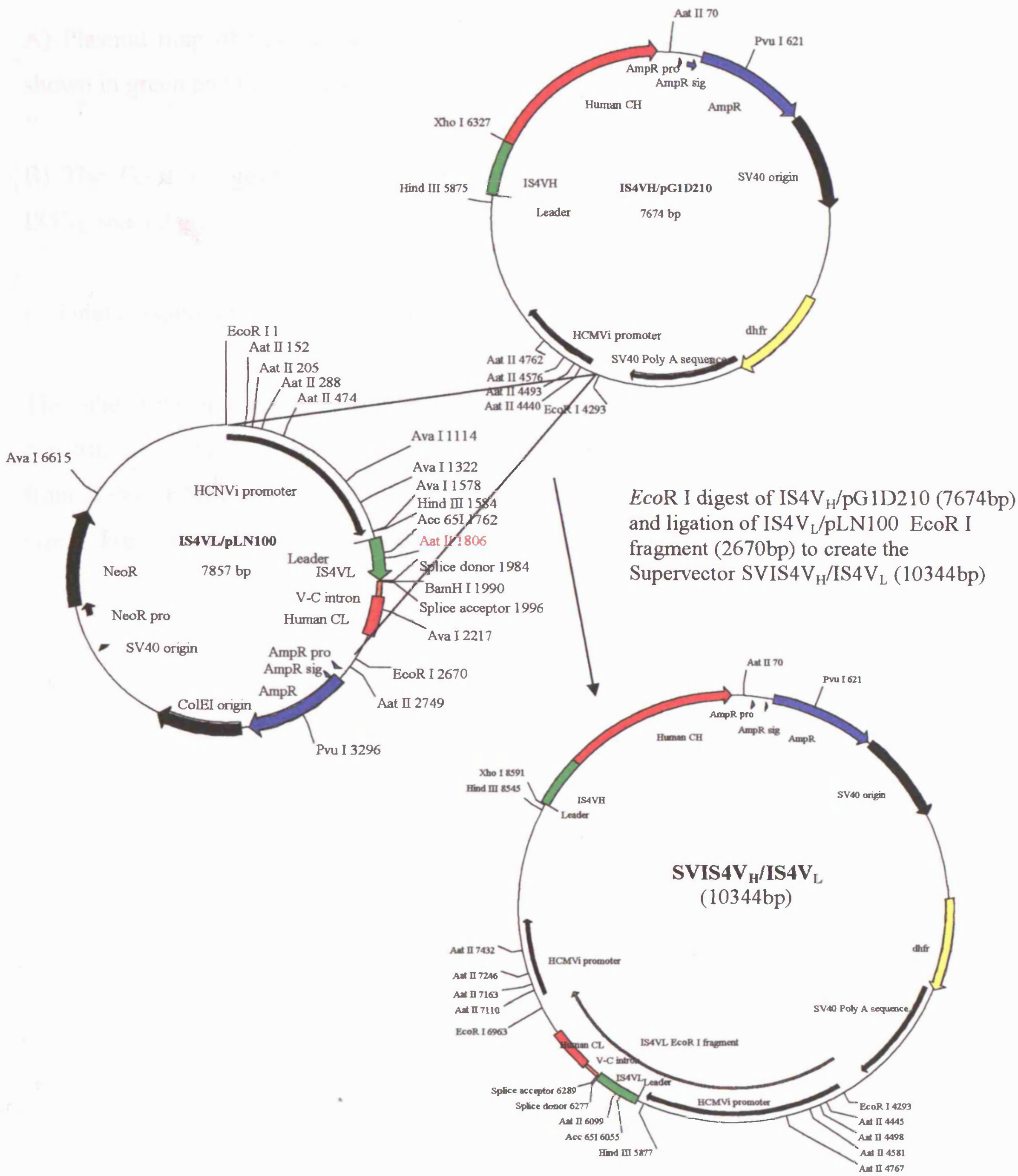


Figure 6.3 Construction of supervectors
For legend see over

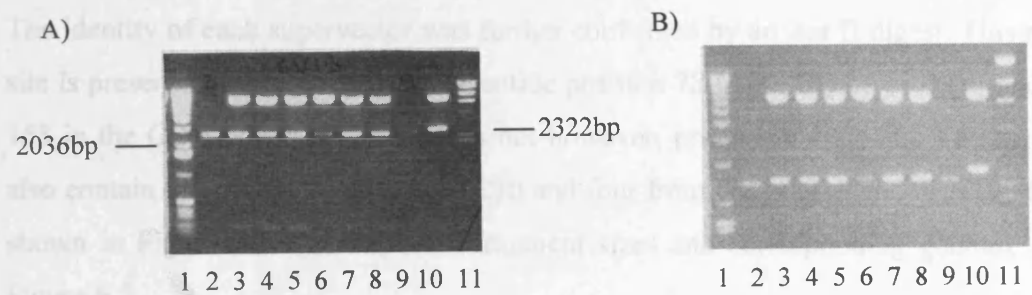
Figure 6.3 Construction of supervectors

A) Plasmid map of IS4V_H/pG1D210 with the *Eco*R I insertion site indicated; IS4V_H is shown in green and the human C_L region in red.

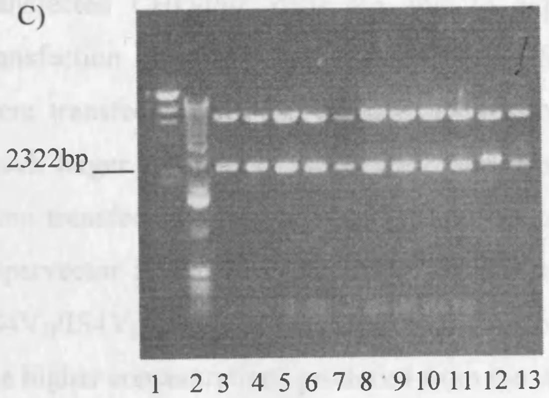
B) The *Eco*R I digestion fragment removed from IS4V_L/pLN100 is shown containing IS4V_L shaded green, the intron shaded brown and the human constant region in red.

C) Final plasmid map of IS4V_H&IS4V_L/pG1D210.

The other two supervectors IS4V_H&B3V_L/pG1D210 and IS4V_H&UK4V_L/pG1D210 were constructed in the same way using the appropriate *Eco*R I digested light chain fragments from B3V_L/pLN10 and UK4V_L/pLN10 leading to slight variations in the overall plasmid size. For a full list of plasmid abbreviations see Figures 2.3 and 2.7.



Lanes in A) and B)
1 = 1Kb marker
2 – 9 = *Hind* III/*Acc65* I digested SVIS4V_H/B3V_L
10 = *Hind* III digested SVIS4V_H/B3V_L
11 = Lambda DNA/*Hind* III marker



Lanes in C)
1 = Lambda DNA/*Hind* III marker
2 = 1Kb marker
3-7 = *Hind* III/*Acc65* I digested SVIS4V_H/UK4V_L
8 - 11= *Hind* III/*Acc65* I digested SVIS4V_H/IS4V_L
12 = *Hind* III digested SVIS4V_H/B3V_L
13 = *Hind* III/*Acc65* I digested SVIS4V_H/B3V_L

Figure 6.4 Confirmation *Hind* III/*Acc65* I digest of supervectors

A) The expected pattern from a *Hind* III/*Acc65* I digest of SVIS4V_H/B3V_L is seen in lanes 3 to 8 with bands of DNA 178bp, 2490bp and 7316bp in size. The Hind III digested SVIS4V_H/B3V_L was run in lane 10 it yields a band of DNA 2668bp in length and serves as a marker for the 2490bp fragment of DNA seen in lanes 3 to 8.

B) The gel displayed in A) was run overnight at 10 Volts. A step is clearly visible between the 2668bp band in lane 10 and the 2490bp bands of DNA in lanes 3 to 9.

C) The expected patterns from a *Hind* III/*Acc65 I* digest are: - 178bp, 2484bp and 7316bp for SVIS4V_H/UK4V_L, seen in lanes 3 to 7; and 178bp, 2490bp and 7676bp for SVIS4V_H/IS4V_L, seen in lanes 8 to 11. Once again the middle bands of DNA for SVIS4V_H/UK4V_L (2484) and SVIS4V_H/IS4V_L (2490) are clearly smaller than the 2668bp band in *Hind* III digested SVIS4V_H/B3V_L in lane 12.

6.3.2 *Aat* II digests

The identity of each supervector was further confirmed by an *Aat* II digest. This restriction site is present in two V_L regions, nucleotide position 78 in the CDR1 of B3V_L and position 158 in the CDR2 of IS4V_L. *Aat* II is not however, present in UK4 V_L. The supervectors also contain five *Aat* II sites in pG1D210 and four from the pLN10 and pLN100 fragments shown in Figure 6.3. The expected fragment sizes and corresponding gels are shown in Figure 6.5.

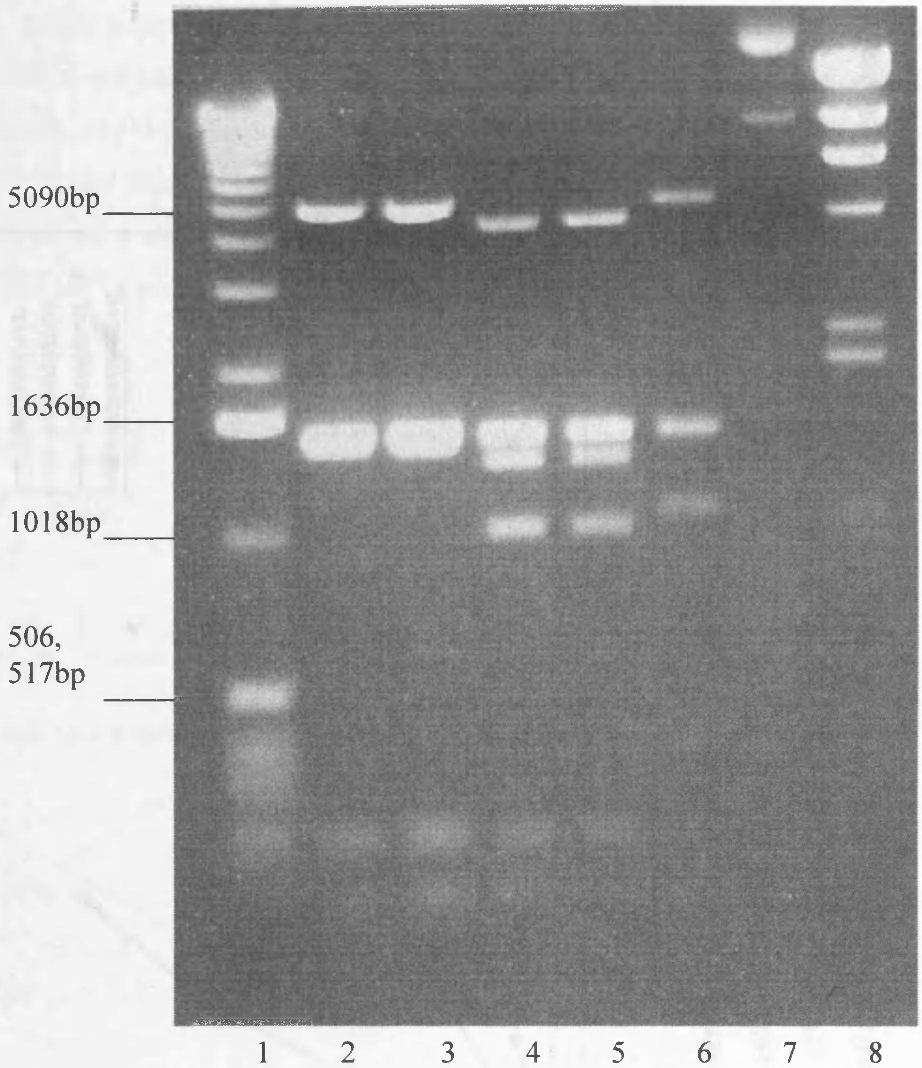
6.4 Detection of whole IgG molecules

6.4.1 Transient expression of whole IgG molecules in COS-7 cells

In the stable expression system it takes 3 – 4 weeks before it can be determined whether the transfected CHO*dhfr*⁻ cells are able to express whole IgG molecules. Thus prior to transfection into CHO*dhfr*⁻ cells SVIS4V_H/IS4V_L, SVIS4V_H/B3V_L and SVIS4V_H/UK4V_L were transfected into the transient expression system. Only SVIS4V_H/UK4V_L produced much larger amounts of whole IgG compared to the range of values previously obtained from transfections with the single chain vectors, shown in Table 6.2. CL binding of each supervector is shown in Figure 6.6. Interestingly SVIS4V_H/IS4V_L binds CL better than IS4V_H/IS4V_L produced by single chain vectors and SVIS4V_H/UK4V_L clearly binds CL at the higher concentrations produced from the supervector.

ANTIBODY	Concentration IgG ng/ml produced in COS-7 cells from:	
	Supervector	Separate heavy and light chain
IS4V_H and IS4V_L	82	24 – 420
IS4V_H and B3V_L	8	4 - 40
IS4V_H and UK4V_L	880	11 - 540

Table 6.2 Concentration of IgG produced by supervectors compared to separate heavy/light chain combinations in COS-7 cells



Lanes
1 = 1Kb marker
2 & 3 = *Aat* II digested SVIS4V_H/UK4V_L
4 & 5 = *Aat* II digested SVIS4V_H/IS4V_L
6 = *Aat* II digested SVIS4V_H/B3V_L
7 = Uncut SVIS4V_H/B3V_L
8 = Lambda DNA/*Hind* III marker

Figure 6.5 *Aat* II digest of supervectors

The expected patterns from an *Aat* II digest were obtained as follows: - for SVIS4V_H/UK4V_L of 53bp, 83bp, 186bp, 1440bp, 1481bp, 1501bp, and 4912bp in lanes 2 and 3; for SVIS4V_H/IS4V_L of 53bp, 83bp, 186bp, 1011bp, 1332bp, 1481bp, 1501bp, and 4375bp in lanes 4 and 5; and for SVIS4V_H/B3V_L of 53bp, 83bp, 186bp, 363bp, 1083bp, 1481bp, 1501bp, and 4912bp in lane 6.

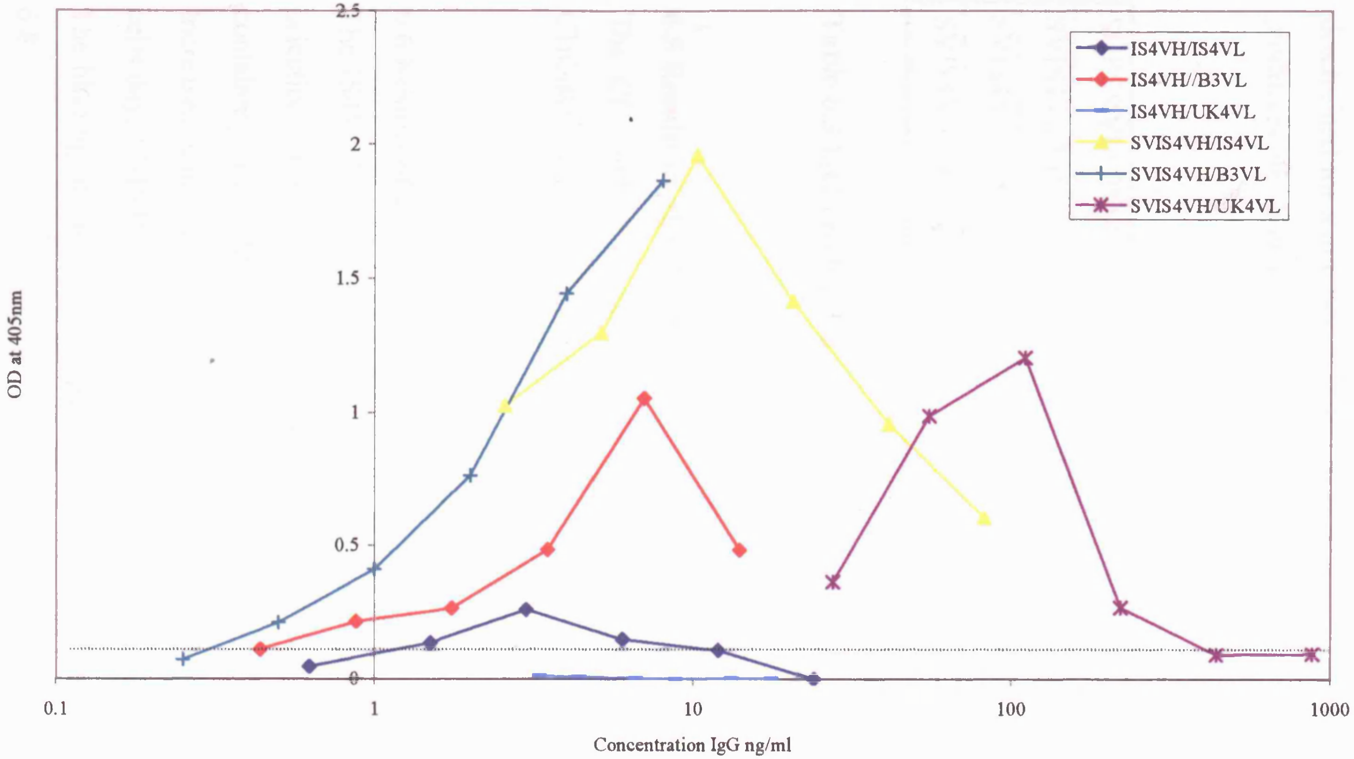


Figure 6.6 Comparison of the cardiolipin binding of three antibodies produced by transfection of separate heavy and light chain plasmids and supervectors in COS-7 cells detected by ELISA

The cardiolipin binding of IgG in COS-7 cell supernatants containing the supervectors SVIS4V_H/IS4V_L, SVIS4V_H/B3V_L and SVIS4V_H/UK4V_L is compared with the cardiolipin binding of the same whole antibodies produced by transfection of separate heavy and light chains.

6.4.2 Stable expression of whole IgG molecules in CHO*dhfr*$^-$ cells

Three stable cell lines were produced from a single round of selection in growth medium B lacking ribo and deoxyribonucleosides (see appendix D). The amount of IgG produced was determined as described in chapter 2.18.1 from which the IgG production rate was determined for a number of clones of each supervector. The production rates of the highest producers are shown in Table 6.3.

SUPERVECTOR	IgG Production rate (ng/10^6 cells/day)
SVIS4V_H/IS4V_L	7
SVIS4V_H/B3V_L	6
SVIS4V_H/UK4V_L	100

Table 6.3 IgG production rates of stable cell lines

6.5 Results of aCL ELISA

The CL binding of each of the IS4V_H/2a2-derived V_L IgG products expressed from CHO*dhfr*$^-$ cells is shown in Figure 6.7.

6.6 Results of anti-β_2GPI ELISA

The IS4V_H/2a2-derived V_L IgG supervector combinations derived from a single round of selection of CHO*dhfr*$^-$ cells described above, were grown in selective growth media containing $1x10^{-8}$M concentrations of methotrexate (MTX) by Nancy Lambrianides. Increased amounts of each supervector were produced: SVIS4V_H/IS4V_L - 144 ng/10^6 cells/day; SVIS4V_H/B3V_L - 40 ng/10^6 cells/day; SVIS4V_H/UK4V_L - 160 ng/10^6 cells/day. The binding of each of the supervectors to β_2GPI was tested by me and is shown in Figure 6.8.

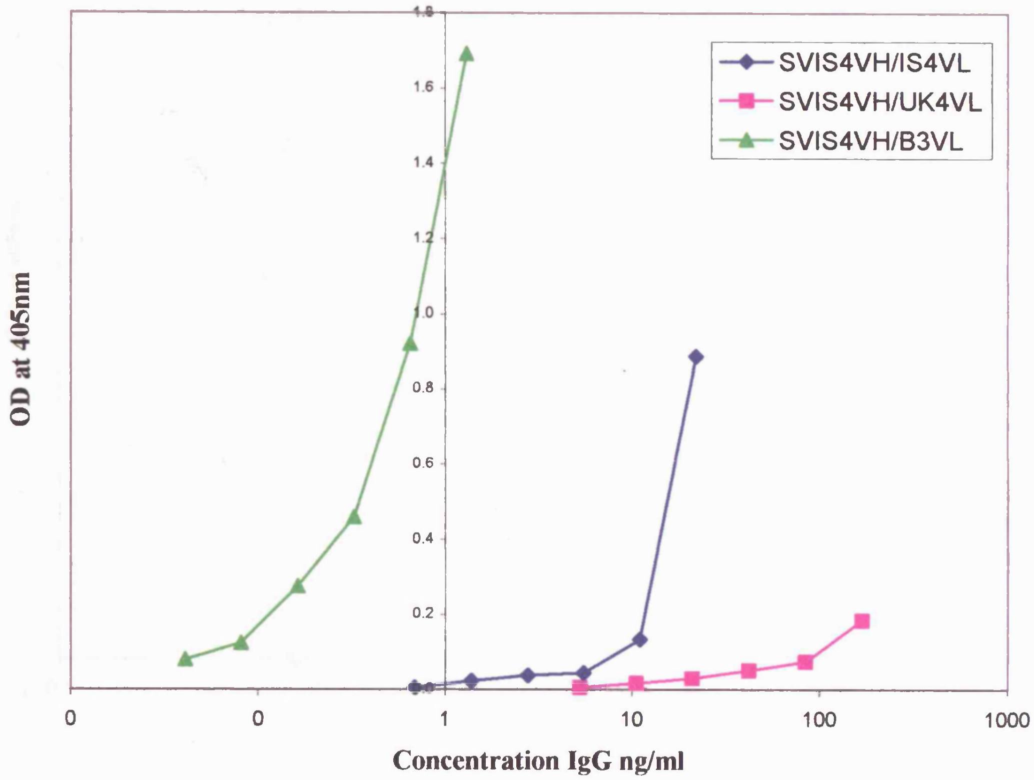


Figure 6.7 Cardiolipin binding of whole IgG produced from CHO*dhfr*⁻ cells detected by ELISA

The cardiolipin binding of IgG produced in CHO*dhfr*⁻ cell supernatants containing the highest producers from each of the three supervector cell lines is shown.

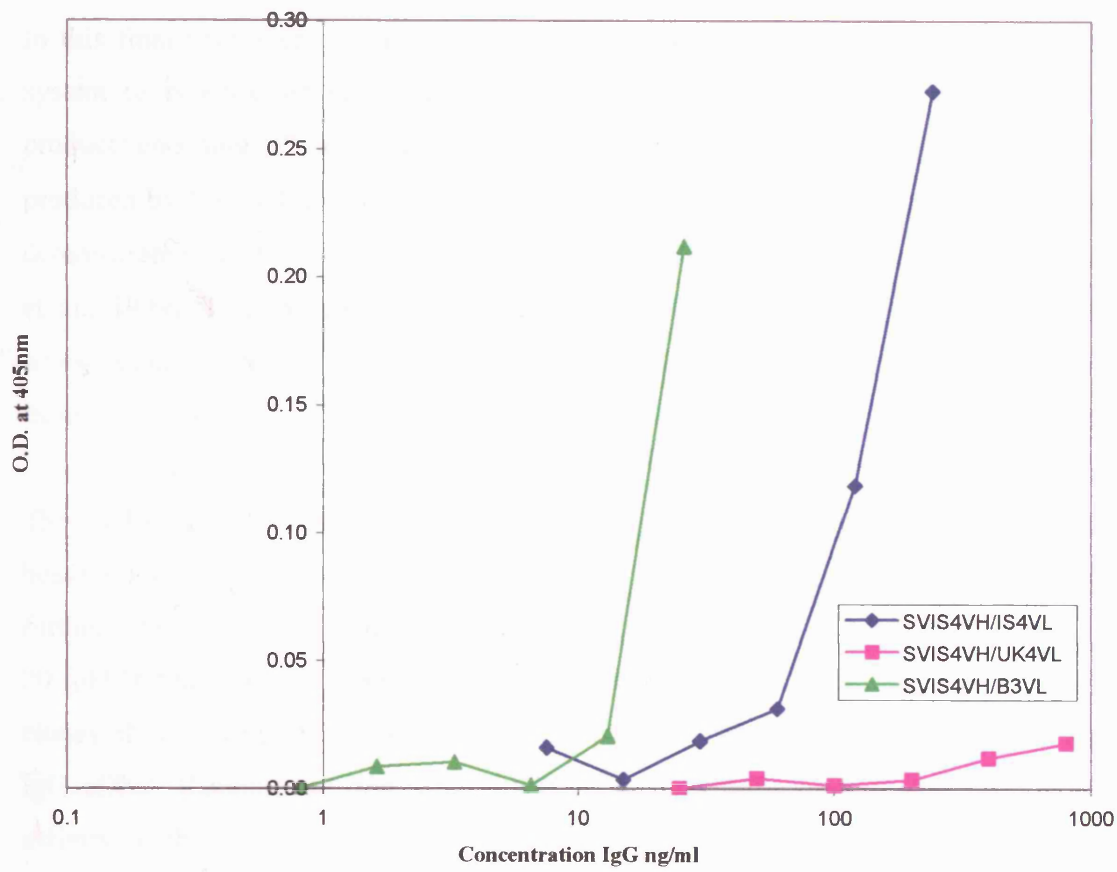


Figure 6.8 β_2GPI binding of whole IgG produced from CHO*dhfr*$^-$ cells detected by ELISA

The β_2GPI binding of IgG produced in supernatants from CHO*dhfr*$^-$ cells following amplification with methotrexate and selection of those lines secreting the highest amount of IgG.

6.7 Discussion of results

In this final results chapter I describe the successful development of a stable expression system to produce whole human recombinant aPL IgG1 molecules from cloned PCR products encoding aPL antibody sequences. Furthermore, when larger amounts of IgG were produced by Nancy Lambrianides using MTX amplification, binding to β_2GPI was clearly demonstrated (see Figure 6.8), thus replicating the observations of the original authors (Zhu et al., 1999). This system has the potential to produce significant amounts of antibody whose sequence-related functional properties can be studied both in *in-vitro* and *in-vivo* assays.

The total yield of IgG1 varied between 6 and 100 ng per 10^6 cells per day depending on the heavy/light chain combination expressed. The yield of these antibodies was increased by further selection of the stable cell lines in selective growth media containing MTX by up to 20 fold in the case of SVIS4V_H/IS4V_L. This process of MTX amplification selects those clones of cells with a higher *dhfr* copy number and consequently a higher copy number of IgG cDNA flanking the *dhfr* gene. MTX is a competitive inhibitor of the intracellular activity of the *dhfr* enzyme and MTX resistance is proportional to the amount of *dhfr* activity and the degree of amplification of the *dhfr* gene. In resistant cells where amplification has occurred there is sufficient free enzyme to generate intracellular tetrahydrofolate required for cell biosynthesis.

The yield of antibody produced by MTX amplification is still very low when compared to the amounts produced by hybridoma cell lines. However, larger yields could possibly be obtained with these cell lines by transferring them into larger scale *in-vitro* culture systems. In fact, a stable cell line of human anti-dsDNA antibodies (B3) produced by Dr J. Haley has been tested in a hollow fibre system by a company (Chemicon) in collaboration with our group and they have produced milligram quantities of purified antibody by this method. With such large amounts of purified antibody, not only could these antibodies be studied *in-vivo*, but more accurate information about changes in affinity for CL and β_2GPI could be obtained in assays such as SPR or inhibition ELISA.

The CL binding properties of the three IgG heavy/light chain combinations after transfection of supervectors into COS-7 cells are similar but not identical to those seen with the same whole IgG1 produced after transfection of single chain vectors into COS-7 cells. SVIS4V_H/B3V_L and SVIS4V_H/IS4V_L displayed stronger CL binding compared to the equivalent aPL from separate heavy and light chain vectors over similar concentration ranges, see Figure 6.6. In particular SVIS4V_H/IS4V_L has a more consistent CL binding pattern when produced from both COS-7 and CHO*dhfr*$^-$ cells.

Heavy/light chain imbalance issues: - a possible explanation for differences in binding properties of antibody derived from transfection of supervectors and separate heavy/light chain vectors arises from the possibility of imbalance in expression of heavy and light chains. A theoretical risk exists, of an imbalance in the production of heavy or light chains when COS-7 cells are co-transfected with two expression vectors. This inequity may occur if more cells take up only one vector rather than both vectors together; or if one of the heavy/light chain genes is expressed at a lower level compared to the other. If such an imbalance in the expression of heavy or light chains were to occur it could affect the binding abilities of the expressed IgG1, since heavy chain fusion proteins (each containing a V_H domain and a B domain of staphylococcal protein A) have been shown to bind dsDNA in the absence of any light chain (Lecerf et al., 1998). Therefore it might be argued that the difference in binding seen with the whole IgG1 produced by supervectors compared to separate expression vectors in COS-7 cells arises as a result of an excess of either free heavy or light chains produced from separate vectors.

There is also a potential risk of an imbalance in heavy/light chain expression with the supervectors due to their design. The construction of the supervectors involves a light chain expression cassette (containing the HCMV promoter, the λ constant region gene and the λ variable region gene of chosen autoantibody) being transferred into the heavy chain expression vector directly upstream from the HCMV promoter, see Figure 6.3. As a result the light chain and heavy chain are being expressed in sequence. When two promoters in sequence are used to express two genes in this way the second gene may be expressed at a lower level than the first. Therefore it is possible that the heavy chain in the supervectors will be expressed at lower levels than the light chain.

There are a number of reasons why I do not think that the results I have described in this thesis are due to heavy or light chain over expression from either separate expression vectors or supervectors. Firstly, in a number of experiments carried out at AERES biomedical and in our laboratory by Dr Joanna Haley (Haley, 2003) have not shown adverse effects of heavy/light chain imbalance using this expression system to produce various different antibodies. Secondly, it is clear that the binding properties of the products expressed by the COS-7 cells and CHO cells in my experiments depended on both the heavy and light chain expressed, which would not be the case if over expression of either chain were the dominant factor. For example IS4V_H has very different binding properties in combination with each of the five light chains paired with it in chapter four, ranging from no binding (IS4V_H/UK4VL) to very strong binding (IS4V_H/B3V_L).

The demonstration of binding to β_2GPI in the absence of CL described in this chapter and shown in Figure 6.8, confirms the observations of Zhu *et al.* (1999) who found that IS4 secreted by hybridoma cells bound β_2GPI more weakly than it bound CL. The fact that IS4 binds β_2GPI is further evidence of the relevance of the study of a single monoclonal aPL (IS4) to try and advance our understanding of the pathogenesis of the APS. Since β_2GPI is recognised as an important cofactor for pathogenic aPL the demonstration that IS4 binds this clinically relevant antigen lends further support to the argument that this aPL is typical of pathogenic aPL found in other patients with APS.

There are very few published reports of the production of functional aPL from bacterial or eukaryotic expression systems using cloned DNA sequences, discussed in chapter 1. Only one report describes a stable expression system to produce a murine monoclonal anti-PC antibody (6G6) as whole IgM molecules secreted from HCLV (Pewzner-Jung et al., 1996). No group has ever reported *in-vitro* stable expression of whole human aPL antibodies with these pathogenic properties.

Therefore the stable expression of IS4 and variants reported here is to my knowledge, the first report of stable expression of human aPL and the future challenge will be to produce sufficient quantities of these whole Ig molecules so that the pathogenic properties of sequence alterations can be determined.

CHAPTER 7

OVERVIEW AND IDEAS FOR FUTURE WORK

Work described in this thesis demonstrates the importance of Arg residues in the binding of both human heavy and light chain combinations to CL, with the heavy chain of IS4 dominant in conferring the ability to bind CL and the nature of the light chain paired with IS4V_H determining the strength of binding to CL. It is therefore now important to examine the functional properties of the variants of IS4, because it does not necessarily follow that an alteration in sequence affecting CL binding will lead to an alteration in function. Experiments to produce mutagenised forms of the V_H of a murine monoclonal anti-dsDNA antibody (including alterations of arginine residues) have shown that sequence alterations which enhanced binding to dsDNA did not always enhance the antibodies' capacity to cause glomerulonephritis when administered to mice (Katz et al., 1994). Similarly, sequence analysis of a range of aPL tested in the *in-vivo* models of microcirculation by Pierangeli *et al.* (2000) revealed that there is no simple relationship between pathogenicity and strength of binding to CL. Two of the antibodies tested (IS1 and IS2), contained identical V_H and their light chains differed from each other by only five amino acids (Chukwuocha et al., 2002). IS1V_L contained slightly more mutations than IS2V_L which improved its reactivity for antigen but abrogated its thrombogenic activity (Zhu *et al.*, 1999; Pierangeli *et al.*, 2000).

To investigate the hypothesis that Arg residues in human monoclonal aPL are important in determining both their binding and pathogenic properties a larger yield of a number of heavy/light chain combinations derived from the pathogenic human monoclonal IgG aPL, IS4 would need to be produced. These combinations would have to differ from each other in sequence at specific points where Arg residues appear to contribute to binding to PL. Milligram quantities of purified antibody could be produced by the transfer of different heavy/light chain combinations from stably transfected CHO*dhfr*$^-$ cell lines into larger scale *in-vitro* culture systems, such as the hollow fibre system used by Chemicon, described in section 6.7. Affinity constants for the binding of the antibodies to ß$_2$GPI, could then be obtained from inhibition ELISA and SPR assay, a more accurate technique than ELISA for measuring small changes in antibody binding affinity. More importantly the properties of these expressed affinity purified aPL could then be tested in assays of biological function.

The heavy/light chain combinations that I would select for stable expression of supervectors, so that larger quantities can be purified, would be the three already made: - IS4V_H with IS4V_L (i.e. wild-type IS4 antibody) IS4V_H/B3V_L, IS4V_H/UK4V_L; and other combinations containing B3V_L or IS4V_L with different variants of IS4V_H, each designed to lose particular Arg from CDR3. IS4V_L, UK4V_L and B3V_L are all encoded by the same germline gene (2a2) hence share 93% homology and differ only in their pattern of somatic mutations many of which are Arg residues. Using these combinations, I would be able to determine the effects of Arg residues at particular positions in V_H and V_L upon binding to CL and β_2GPI as well their functional effects in two biological assays.

Currently one of the best animal models in which to demonstrate a thrombogenic effect of human aPL is the *in-vivo* model of microcirculation developed by Pierangeli *et al.* (2000). IS4 has consistently been shown to be highly thrombogenic and to enhance endothelial activation in these *in-vivo* models (Pierangeli *et al.* (2000) and (2001)). I am developing a collaboration with Professor Pierangeli to spend time in her laboratory in order to test the effects of my expressed wild-type and variant IS4 molecules upon thrombus formation and leucocyte adhesion *in-vivo.* Furthermore Professor Pierangeli has the facilities to study the effects of aPL upon vascular EC activation *in-vitro* by examining the expression of adhesion molecules with murine monoclonal antibodies against endothelial (E)-Selectin, ICAM-1 and VCAM-1 (Pierangeli et al., 2000).

The effects of IS4 on vascular function have also been studied in organ chamber experiments at UCL (by Dr J. Alves, personal communication). Isolation of a blood vessel with IS4 leads to inducible nitric oxide synthase (iNOS) production in the vessel wall which results in hyporeactivity to vasoconstrictors such as phenylephrine, which can be reversed by nitro-L-arginine methyl ester and 1400W (non-selective and iNOS selective inhibitors respectively). The effects are abolished by pre-isolation with a 100 fold excess of cardiolipin. NO causes hyporeactivity but protects against thrombosis. A lack of NO is likely to occur after the original increase, due to suppression of eNOS as a result of the iNOS activity. The eNOS activity remains low after the iNOS burst ends, leading to a period of low NO which could lead to platelet activation, and thus to thrombosis.

It would be fascinating to examine the effects of incubation of blood vessels with wild type IS4 compared with the variant forms of IS4 described above upon NO production. If alterations in the heavy or light chain of IS4 altered its effects on NO production, the experiment could be repeated to determine whether the alteration is abolished by pre-incubation with cardiolipin or $ß_2$GPI. If so, it would imply that the effect is mediated via changes in the antigen/antibody interaction.

Thus this future research would help to elucidate the precise role of Arg residues in the binding of aPL to their target antigen but also provide greater understanding of how manipulation of aPL binding sites may affect the function of these antibodies and their effects upon endothelium leading to thrombosis and AS. In the future this knowledge could lead to the development of a more targeted therapy designed to block the aPL-$ß_2$GPI-PL interaction thus providing a safer, more effective treatment for APS patients than is currently available.

APENDICES

APPENDIX A ONE LETTER CODE FOR AMINO ACIDS

ONE LETTER CODE	AMINO ACID
A	Alanine
C	Cysteine
D	Aspartic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

APPENDIX B CHEMICALS, MATERIALS AND EQUIPMENT

1) Chemicals

All chemicals used in this project were supplied by BDH, Lutterworth, Leicestershire, UK, with the following exceptions:-

^{35}SdATP	Amersham Pharmacia, Buckinghamshire, UK
RNAzol B	Biogenesis, Poole, Dorset, UK
Absolute Alcohol & Propan-2-ol	Hayman Ltd, Witham, Essex, UK
Dimethyl Sulfoxide	# D5879, Sigma, Poole, UK
Trypan Blue Solution (0.4%)	# T8154, Sigma, Poole, UK
Hanks Balanced Salt Solution (HBSS)	Invitrogen, Paisley, U.K.
Diethyl pyrocarbonate (DEPC) Solution	Sigma, Poole, UK

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in dimethylformamide (Xgal) and Isopropyl – β – thiogalactopyranoside (IPTG) - Insight Biotechnology Ltd, Wembley, U.K.

All solid chemicals were dissolved in ddH_2O, adjusted to the correct pH with 0.1M HCL, glacial acetic acid or 0.1M NaOH and autoclaved or filter sterilised unless otherwise stated. NaOH was not autoclaved. Ethidium bromide was stored in the dark.

2) General materials and equipment

15ml and 50ml Sterile tubes	# 91015T, Helena Biosciences, Sunderland, U.K.
Bijou tubes	# 39740, Bibby Sterilin Ltd, Staffordshire, U.K.
Sterilin tubes	# 30593, Bibby Sterilin Ltd.
1.5ml and 0.5ml microfuge tubes	Anachem, Luton, Bedfordshire, U.K.
Centrifuges	Sorvall Biofuge Pico (and refrigerated version), Sorvall RT-7 Plus and RC26 Plus, Kendro Laboratory Products Ltd, Herts, U.K.
PCR Machine	Eppendorf Mastercycler, Hamburg, Germany
Gene Pulser II Electroporator	Bio-Rad Laboratories Ltd, Herts, U.K.
Gene Pulser II Cuvettes 0.4cm	# 165-2088, Bio-Rad Laboratories Ltd

APPENDIX C ENZYMES, BUFFERS AND SOLUTIONS

Enzymes

All restriction enzymes, DNA modifying enzymes, RNAse inhibitor and buffers were supplied by Promega, Southampton, U.K. with the following exceptions:-

Sfi I and *Bam*H I	New England BioLabs (NEB), Herts, UK
DNA Polymerase (Sequenase version 2.0)	USB Corporation, Cleveland, USA
Rnase-free DnaseI	#776785, Roche, Lewes, East Sussex, U.K.
PfuTurbo DNA Polymerase	Stratagene, California, U.S.A.
Phospholipase C	# P4039, Sigma, Poole, U.K.

All enzymes and reaction buffers were stored at -20°C.

Reaction buffers

a.) PCR Buffer 10x (Promega)	50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C) 1.5mM $MgCl_2$, 0.1% Triton®X-100
b.) Ligase Buffer 10x (Promega)	300mM Tris-HCl (pH 7.8), 100mM $MgCl_2$ 100mM DTT, 10mM ATP
c.) 1X NEBuffer for *BamH I*(NEB)	150mM NaCl, 10mM Tris-HCl (pH 7.9 at 25°C) 10mM $MgCl_2$, 1mM DTT
d.) 1X NEBuffer 2 for *Sfi*1(NEB)	50mM NaCl, 10mM Tris-HCl (pH 7.9 at 25°C) 10mM $MgCl_2$, 1mM DTT

e.) Buffers A, B, D, E, H and J for restriction digests (Promega)

f.) Sequenase 5x reaction buffer (USB)

h.) 10x *PfuTurbo* DNA Polymerase reaction buffer (Stratagene, California, U.S.A.) 100mMKCl, 100mM $(NH_4)_2SO_4$, 200mM Tris-HCl (pH 8.8), 20mM $MgSO_4$, 1% Triton X-100, 1mg/ml nuclease free bovine serum albumin

General buffers and solutions

Phosphate buffered saline (PBS) pH 7.4. One PBS tablet (Invitrogen) was added per 500ml of sterile water. To make PBS/0.1% Tween, 1ml "Tween 20" detergent was added to 1l PBS

Tris-EDTA (TE) buffer, pH7.5 10mM Tris-HCL (pH7.5), 1mM EDTA

Specific buffers and solutions

a) Agarose gel electrophoresis

1 x Tris Acetate EDTA (TAE buffer)	40mM Tris base, 20mM glacial acetic acid, 1mM EDTA (pH 8)
DNA markers	1Kb DNA MW marker (Invitrogen.) Lambda/*Hind* III DNA MW marker (Promega)
Loading Buffer	0.25% (w/v) xylene cyanole, 1.5% (w/v) Ficoll 400 (Pharmacia, Biotech, Uppsala, Sweden) in ddH_20 stored at room temperature

b) Purification of amplified V_H and V_L cDNA from agarose gels

New Wash buffer 14ml of concentrated NaCl, Tris and EDTA (#1001-402) solution, 280ml ddH2O and 310ml 100% Ethanol

c) DNA extraction from bacterial cultures

Buffer P1	(Resuspension Buffer)	50mM Tris-Cl, pH 8.0, 10mM EDTA 100μg/ml Rnase A
Buffer P2	(Lysis Buffer)	200mM NaOH, 1% SDS
Buffer N3	(Neutralisation Buffer)	3.0mM potassium acetate, pH 5.5
Buffer QBT	(Equilibriation Buffer)	750mM NaCl, 50mM MOPS, pH 7.0 15% isopropanol, 0.15% Triton®X-100
Buffer QC	(Wash Buffer)	1.0M NaCl, 50mM MOPS, pH 7.0 15% isopropanol
Buffer QF	(Elution Buffer)	1.25M NaCl, 50mM MOPS, pH 7.0 15% isopropanol

d) Polyacrylamide gel electrophoresis

Tris Borate EDTA (TBE) buffer	45mM Tris borate, 1mM EDTA (pH 8.0)
Sequencing gel	60ml polyacrylamide gel solution (Amresco,Ohio, USA), 300μl 10% (w/v) ammonium persulphate solution, 30μl N, N, N`, N` tetramethylethylenediamine (Sigma)

e) ELISA Reagents and buffers

Bovine serum albumin (BSA)	# A7030, Sigma.
Goat anti-human IgG (Fc fragment specific)	# I8885, Sigma.
Cardiolipin	# C1649, Sigma.
B2-Glycoprotein I	# B2G1-0001, Haematologic Technologies Inc, U.S.A.
Purified human IgG_{λ}	# I5029, Sigma.
Goat anti-human λ alkaline phosphatase conjugate	# A2094, Sigma.
Goat anti-human κ alkaline phosphatase conjugate	# A3813, Sigma.
Goat anti-human IgGγ alkaline phosphatase conjugate	# A3150, Sigma.
p nitrophenyl phosphate tablets	# 104-105, Sigma.

Sample, enzyme and conjugate (SEC) buffer

100mM Tris-HCl (pH 7), 100mM NaCl, 0.02% Tween 20 and 0.2% BSA.

Bicarbonate (BIC) buffer

0.5M sodium bicarbonate and 0.05M sodium dihydrogencarbonate.

APPENDIX D ANTIBIOTICS AND GROWTH MEDIA

Bacterial growth media

Media were made in ddH_2O and sterilised by autoclaving at 121°C for 15 minutes at 15lb/inch^2.

Luria-Bertani (LB) medium	1% (w/v) Bacto-tryptone (Duchefa, The Netherlands) 1% (w/v) NaCl, 0.5% (w/v) Bacto-yeast extract (Duchefa). Adjusted to pH 7.0 using 5M NaOH

Agar plates were prepared from this by the addition of 1.5% (w/v) agar (Duchefa)

NZY Medium	1% (w/v) NZ Amine (Sigma), 5% (w/v) Bacto-yeast extract (Duchefa), 5% NaCl plus filter sterilised 1.25% 1M $MgCl_2$, 1.25% 1M $MgSO_4$ and 1% 2M Glucose prior to use.

Where antibiotics were added to media and LB agar plates final concentrations of 50µg/ml ampicillin or 50µg/ml chloramphenicol were added. Stock solutions of ampicillin at 50mg/ml in sterile water and chloramphenicol at 34mg/ml in ethanol were kept at -20°C in light-tight containers.

Growth media and solutions for maintenance of eukaryotic cell lines

COS-7 growth medium 1 (Pre-Electroporation)

Dulbecco's Modified Eagle Medium (DMEM) (41966-029, Invitrogen, Paisley, U.K.) containing 10% (v/v) Foetal calf serum (FCS) (10099-133, Invitrogen), 580µg/ml L-glutamine (25030-024, Invitrogen), 10000 Units/ml penicillin / 10mg/ml streptomycin (15140-122, Invitrogen).

COS-7 growth medium 2 (Post-Electroporation)

As for medium 1 except FCS was replaced by Ultra low IgG FCS (#16250-078, Invitrogen).

COS-7 growth medium 3 (Post-Electroporation)

As for medium 1 except FCS was replaced by 2% (v/v) Ultroser G serum replacement (#15950-017, Invitrogen),

CHO*dhfr⁻ (Non-selective) growth medium A*

MEM α-Medium (α-MEM) with ribonucleosides and deoxyribonucleosides (#32571-028, Invitrogen) containing 10% (v/v) FCS (#10099-133, Invitrogen), 5000 Units/ml penicillin / 5mg/ml streptomycin.

CHO*dhfr⁻ (Selective) growth medium B*

MEM α-Medium (α-MEM) without ribonucleosides and deoxyribonucleosides (#32571-029, Invitrogen) containing 10% (v/v) Dialysed FCS (#FH 30079, Hyclone), 5000 Units/ml penicillin / 5mg/ml streptomycin.

Hanks` Balanced Salt Solution, without magnesium and calcium (HBSS) (#14175-053, Invitrogen).

10% Trypsin (#25090028, Invitrogen) in Versene (#15040033, Invitrogen)

APPENDIX E MAIN PUBLICATIONS ARISING FROM WORK DESCRIBED IN THIS THESIS

Relative importance of different human aPL derived heavy and light chains in the binding of aPL to cardiolipin. I.Giles, J.Haley, S.Nagl, D.Latchman, P.Chen, R.Chukwuocha, D.Isenberg and A.Rahman. Molecular Immunology (2003) 40, 49-60.

A systematic analysis of sequences of human antiphospholipid antibodies (aPL) and anti-β2Glycoprotein I antibodies reveals the importance of somatic mutations and certain sequence motifs. I.Giles, J.Haley, S.Nagl, D.Isenberg, D.Latchman and A.Rahman. Seminars in Arthritis and Rheumatism (2003) 32, 246-65.

How do antiphospholipid antibodies bind phospholipid? I.Giles, D.Isenberg, D.Latchman and A.Rahman. Arthritis and Rheumatism (2003) 48, 2111-21.

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Somatic mutations to Arginine residues affect the binding of human monoclonal antibodies to DNA, Histones and SmD. J.Haley, L.Mason, S.Nagl, I.Giles, D.Latchman, D.Isenberg, and A.Rahman. (2003) Molecular Immunology (2003) 40, 745-758.

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